The Dissertation Committee for Xiaogang Cheng Certifies that this is the approved version of the following dissertation:

F exclusion of bacteriophage T7

Committee:

Ian J. Molineux, supervisor

Charles F. Earhart

Shelley M. Payne

Richard J. Meyer

James J. Bull

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by

Xiaogang Cheng, D.V.M., M.S.

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Xiaogang Cheng, PhD

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Supervisor: Ian J. Molineux

T 7 fails to productively infect *E. coli* cells containing the plasmid F, a phenomenon now referred to as F exclusion. The F protein PifA and the T7 proteins gp10 and gp1.2 are known to be responsible for exclusion. Co-expression of *pifA* and gene *1.2* or *pifA* and gene *10* is known to be lethal to *E. coli*; selecting for survivors of a lethal challenge *pifA* and gene *1.2* resulted in the isolation of a strain containing the *fxsAp109* mutation. This mutation leads to overproduction of the inner membrane protein FxsA. The same mutation had been isolated previously by selecting survivors of *pifA* and gene *10* co-expression, suggesting that both combinations, PifA plus gp10, and PifA plus gp1.2, attack

the same or similar cellular function. FxsA is not this function, rather, overexpression of *fxsA* serves a protective role.

PifA has been shown to interact with gp10 (Kd=0.3μM) and gp1.2 (Kd=1μM), whereas mutant gp10 and gp1.2, synthesized by mutant phages that escape F exclusion, bind with dramatically reduced affinity. FxsA also interacts with PifA, gp10A and gp1.2. It was shown that gp1.2 and FxsA bind to the of C-terminal 254 residues of PifA while gp10A interacts with the region between residues 181 and 463.

PifA fractionates with the cytoplasmic membrane of *E. coli* but may only be membrane-associated, whereas FxsA was shown to possess four transmembrane segments. Both the N- and C-termini of FxsA are cytoplasmic but the hydrophilic C-terminus is not required for FxsA function; conversely, the fourth transmembrane segment of FxsA is essential for function. As gp10A and gp1.2 are localized in the cytoplasm, these proteins likely interact with FxsA and PifA at the cytoplasmic membrane. This membrane is therefore the site within *E.coli* where F exclusion of T7 is initiated.

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Chapter 1: Introduction

Extrachromasomal parasites of bacterial cells such as plasmids and prophages have evolved various mechanisms by which they maintain their territory and protect their hosts from certain other infecting bacteriophages or colonizing plasmids. Plasmids and prophages can protect their host in a variety of ways that include conferring immunity to superinfection by homoimmune phages, altering specific receptors to block phage adsorption, preventing DNA ejection by adsorbed phage, and degrading unmodified DNA that does enter the cell. Less well understood are those phage infections that abort midway through their infection cycle. During an abortive infection, the individual infected cell dies before producing progeny phage, thereby allowing the overall survival of a bacterial population. A common characteristic of abortive infections is that initial events appear normal, but thereafter a plethora of physiological defects are observed that, in many cases, lead to a cessation of all macromolecular synthesis and other processes in the cell. The severity of phage exclusion is often affected by both the genetic background and the physiological state of the restrictive host cell. These observations suggest that either expression of the exclusion functions or their interactions with cellular components vary during cell growth. Abortive infections thus provide model systems for studies of the interactions between a host and its parasite.

A large number of phage exclusion systems are known and some have been extensively studied. Examples include exclusion of T4 *r*II mutants by λ lysogens, exclusion of T7 by the F plasmid, exclusion of T5 and BF 23 by the Col lb plasmid, and exclusion of T4 by *E. coli lit* (Con) strains. Because extensive knowledge on the genetics, biochemistry and biology of T7 has been accumulated, the abortive infection of T7 provides a convenient system to study the interactions between a host and its parasites. The physiological defects associated with each system overlap substantially and there may be a common mechanism of exclusion. Therefore, it is likely that an elucidation of one system will help us to understand the others.

Productive infections by bacteriophage T7

A T7 phage particle consists of an icosahedral capsid (icosahedral symmetry, T=7) with a short, non-contractile tail (Fig. 1). The phage capsid contains 415 molecules of gp10A or gp10B (Steven and Truss, 1986). At one vertex of the icosahedron, 12 molecules of gp8 serve as the head-tail connector for the phage tail; the latter is comprised of 6 copies of gp12 and 12 copies of gp11. Gp6.7 is also likely part of the tail but its precise location and stoichiometry have not been determined. Six tail fibers, each composed of trimers of gp17, are attached to the tail just below its junction with the head-tail connector protein. Three major internal proteins, gp14 (18 copies), gp15 (12 copies) and gp16 (3

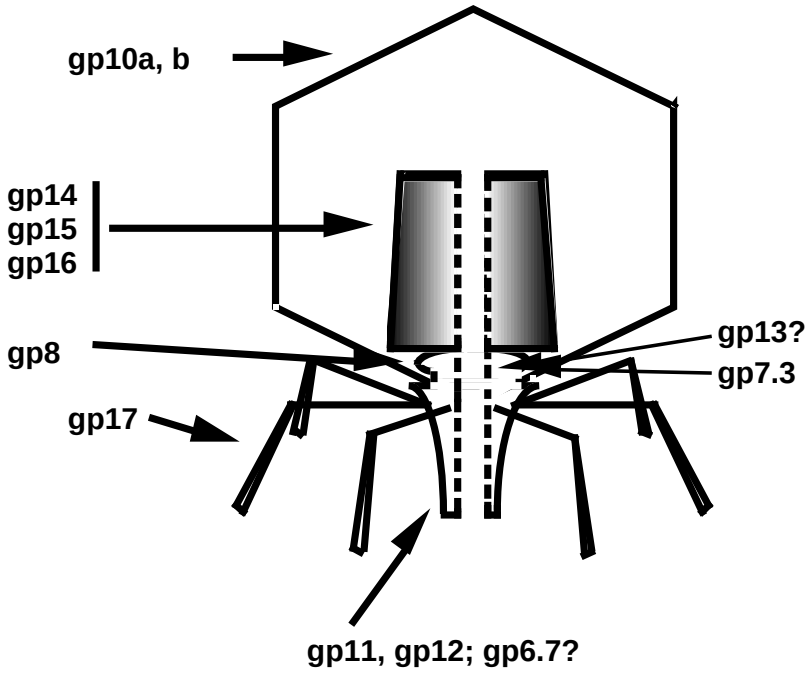
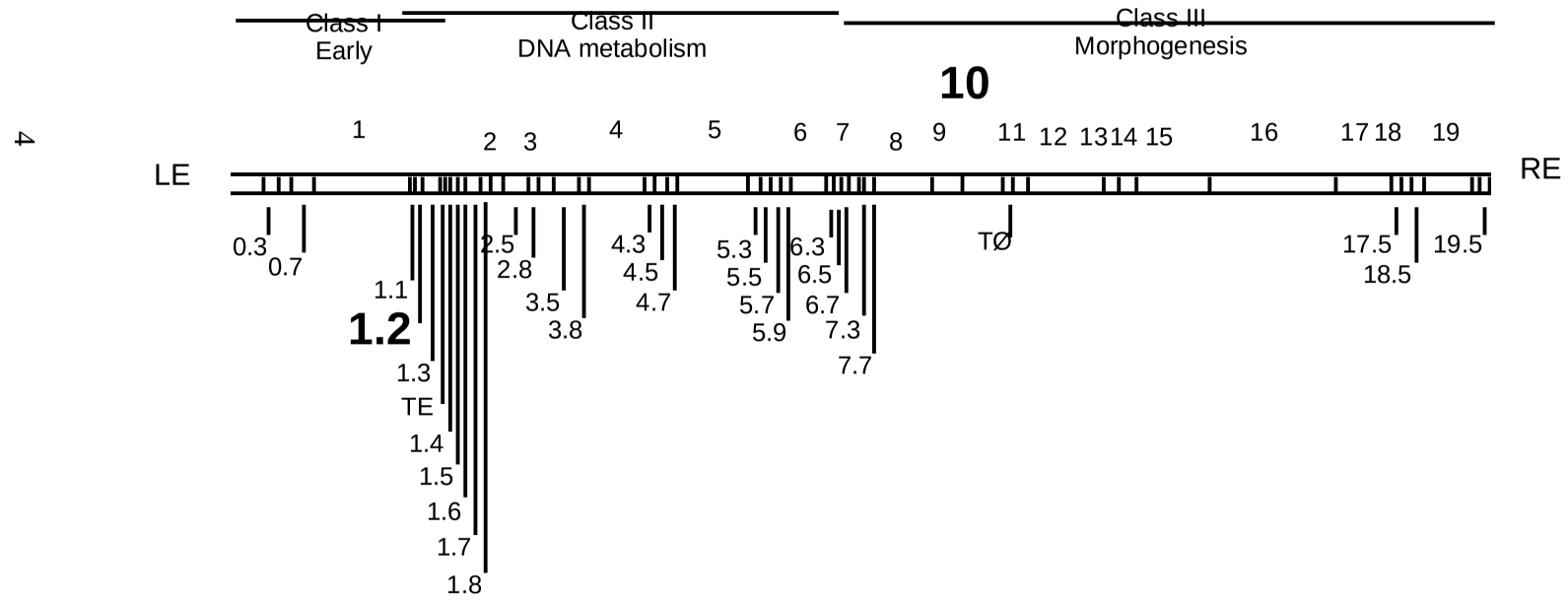


Figure 1. Bacteriophage T7 particle

Figure 2. Genetic map of bacteriophage T7



copies), constitute a hollow cylindrical core structure that is attached to the inner surface of the capsid and the head-tail connector (Steven and Truss, 1986; 1986; Serwer, 1976). This internal core is essential for both phage morphogenesis and DNA ejection (Cerritelli *et al*., 1997). Other head proteins include gp7.3, and perhaps both gp13 and gp7, but their functions are not well-described.

The T7 genome is a linear-double-stranded DNA molecule of 39,937 base-pairs that is terminally redundant but not circularly permuted (Ritchie *et al.*, 1967). Originally, 19 genes were defined genetically, these were assigned with integral numbers sequentially from left to right of the genome beginning at gene *1* (Studier, 1969). As additional genes were discovered they were given fractional numbers in accordance with their positions (Fig. 2). Now, 56 genes are predicted although only 39 gene products have been detected (Dunn and Studier, 1983). Less than half of the genes are essential for phage growth on laboratory strains.

Three classes of genes have been identified. Ten class I, or early, genes are clustered to the left-most 19% of the genome and are transcribed by *E. coli* RNA polymerase. They encode functions that include inhibition of the host type I restriction system, inactivation of host transcription, conversion of host metabolism to the production of T7 phage and a new RNA polymerase. Class II genes, located between 15% and 46% of the genome, and class III genes, located in the right half of the genome, are both transcribed by T7 RNA polymerase. Class

II genes primarily function in T7 DNA metabolism whereas class III genes are involved in phage morphogenesis and maturation or in cell lysis.

Adsorption of T7 to *E. coli* is initiated by the attachment of its tail fibers to the lipopolysaccharide of the host cell outer membrane. Following adsorption, a signal is transduced into the phage head resulting in the ejection of gp6.7 and gp7.3 into the outer membrane. The internal core structure then disaggregates and its components (gp14, gp15 and gp16) also enter the cell through the connector and tail. Gp14 becomes localized in the outer membrane; gp15 and gp16 span the periplasm and the cytoplasmic membrane, and together the three proteins form a transmembrane channel for DNA translocation. The N-terminal region of gp16 is homologous to the soluble lytic transglycosylase of *E. coli* and may enlarge a hole through the peptidoglycan layer. This activity of gp16 is important only when cells are at high density or low temperature, conditions likely favoring increased cross-linking of peptidoglycan (Moak and Molineux, 2000).

Once the transmembrane channel has formed, the phage genome can leave the capsid. About 850 bp at the left end of the genome, containing the *E. coli* promoters designated A1, A2 and A3, are brought into the cell by a transcription-independent mechanism (Moffatt and Studier, 1988; Garcia and Molineux, 1995). Translocation of the leading end of the genome into the host cell is thought to be driven by an enzymatic motor, likely consisting of gp16 and gp15, and to be powered by the proton motive force. After entry of ~850 bp, this motor stops. *E.*

coli RNA polymerase then transcribes the class I genes (Minkley and Pribnow, 1973; Dunn and Studier, 1983) and in doing so pulls the first 19 % of the genome into the cell (Garcia and Molineux, 1995). Most early transcription terminates at the rho-independent early terminator TE, located between gene *1.3* and *1.4* (Dunn and Studier, 1980). TE is not wholly efficient, and readthrough transcripts terminate, also inefficiently, near the 3' end of gene *3.5*, distal to *10* at the T7 RNA polymerase terminator, or at the end of the genome.

There are five RNase III recognition sites in the class I polycistronic mRNA. These early transcripts are cleaved to form shorter messages, most of which are still polycistronic (Dunn and Studier, 1973; Robertson *et al*., 1977). The processed transcripts are then heavily translated. Between 2 and 4 min after infection, the class I gene products gp0.3, gp0.7 and gp1 become detectable (Studier, 1972). During this period, gp0.3 binds to and inactivates host type I restriction enzymes (Studier, 1975; Mark and Studier, 1981; Bandyopadhyay *et al*., 1985); gp0.7, a serine/threonine-specific, cAMP-independent protein kinase, shuts down all host transcription and thus indirectly directs the translation machinery to phage mRNAs (Rothman-Denes *et al*., 1973). The latter become abundant as gp1, T7 RNA polymerase, begins to transcribe class II and class III genes (Chamberlin *et al*., 1970; Summers and Siegel, 1970). As the synthesis of class II gene products begins, gp0.7 shuts off class I gene transcription (Rothman-Denes *et al*., 1973; Rahmsdorf *et al*., 1974; Hesselbach and Nakada, 1977).

Synthesis of class II mRNA is initiated from ten class II promoters (Dunn and Studier, 1983). The majority of transcripts from these promoters terminate at Tø, located just downstream of gene *10*. T7 RNA polymerase reads through Tø about 20% of the time and readthrough transcripts stop near the genome end (Niles and Condit, 1975; McAllister and McCarron, 1977; McDonald *et al*., 1994). Class II mRNAs are also processed by *E. coli* RNase III. Between 4 and 6 min post-infection, class II proteins are first detected (Studier, 1972). The majority of class II proteins are involved in phage DNA metabolism. Gp3 (endonuclease) and gp6 (exonuclease) digest the host genome into nucleotides that are then used for phage DNA synthesis (Center *et al.*, 1970; Sadowski and Kerr, 1970; Kerr and Sadowski, 1972). Gp2.5 (single-stranded DNA-binding protein), gp4A (helicase + primase activity), 4B (helicase activity) and gp5 (DNA polymerase) are directly involved in synthesizing T7 DNA. 15 minutes after infection, class II protein synthesis is normally shut-off by an unknown mechanism.

Class III proteins are synthesized from 6 and 8 minutes post-infection until cell lysis, which occurs 25-30 min post-infection at 30°C. During this period, while class III genes are being heavily transcribed and their mRNAs are rapidly translated, class II transcription is selectively turned off. T7 lysozyme (gp3.5) binds to T7 RNA polymerase and prevents its isomerization from the initiation to elongation complex (Zhang and Studier, 1997). Although the class III promoters are more efficient at initiating transcription than the class II promoters on linear

templates, the inhibition by T7 lysozyme shows no promoter preference *in vitro* (Ikeda, 1992; Ikeda and Bailey, 1992). Class II promoters produce less mRNA, mainly because of an increased frequency of abortive initiation, which is further enhanced by formation of a complex of T7 RNA polymerase and lysozyme (Zhang and Studier, 1997; Lyakhov *et al*., 1998). Abortive initiation is the main reason why class II promoters appear weaker than class III promoters, and why class II gene expression is selectively shut-off during infection.

T7 DNA synthesis starts about 10 min post-infection and, by measurements of thymidine incorporation, stops at cell lysis (Studier, 1972). However, most of the increase in T7 DNA occurs between about 15 to 20 minutes. The ø1.1A and ø1.1B T7 promoters, located 15% from the left end of the genome, serve as origins of replication (Tamanoi *et al.*, 1980; Saito *et al.*, 1980). T7 RNA polymerase initiates DNA replication by synthesizing primers of 10-60 nucleotides from both promoters. T7 DNA polymerase (gp5 + thioredoxin) then displaces T7 RNA polymerase from DNA, and a gp4A and gp4B complex assembles to form a hexameric donut shape structure on the single-stranded DNA at the opened replication origin (Engelman et al., 1995). Gp4 then translocates 5’ to 3’ to unwind unreplicated DNA using ribo- or deoxy-NTP hydrolysis for energy (Hingorani *et al*., 1997). The helicase interacts with both gp2.5 (single-stranded DNA-binding protein) and DNA polymerase to make DNA synthesis more processive. Replication proceeds bi-directionally on the linear genome (Fuller and Richardson,

1985), and synthesis on the leading and lagging DNA strands is tightly coupled by the gp4 complex (Lee *et al*., 1998).

The replicated DNA molecules connect to each other in a left end to right end fashion via the terminal repeat to form linear concatemers. As replication proceeds, linear concatemers are converted into highly branched DNA through homologous recombination. Recombination requires gp2.5, gp4, gp3 and gp6, but is independent of the host recombination system (Powling and Kinppers, 1974; Kerr and Sadowski, 1975; Kong and Richardson, 1996). Eventually, the complex of concatemers containing more than 100 genome equivalents is converted by gp3 (Holliday junction resolvase) into linear concatemers that are substrates for the packaging machinery (De Massy *et al*., 1987; Kong and Richardson, 1996; Kong *et al*., 1997).

As DNA replication progresses, the phage head begins to be assembled. Assembly may be initiated by polymerization of gp10 around the scaffolding protein gp9 to generate an incomplete prohead shell. Insertion of the connector-core complex (gp8-[gp14+gp15+gp16]) into the shell may then follow (Cerritelli and Studier, 1996). Alternatively the connector-core complex may serve to nucleate assembly of the scaffolding and capsid protein. During this process, gp8 would be added to one vertex of the icosahedral procapsid to form the portal. Concurrent with the maturation of the procapsid, DNA packaging initiates from a genomic right end and proceeds leftwards in a process whose details are not well

understood. A series of reactions is necessary in order to duplicate the terminal repeat sequence of T7 DNA that in concatemers is present in only one copy between genomes. A single-strand break is first made in a cruciform generated from a palindromic sequence located to the left of the right terminal repeat (Chung *et al*., 1990). The resulting hairpin end is used to initiate DNA synthesis as a leading strand of a bi-directional replication fork. This fork could then proceed rightward through the terminal repeat and into the genome being packaged, thereby providing duplex DNA that can be converted into a mature left end. On the remaining concatemeric DNA, primase-initiated synthesis on the displaced strand could proceed through the terminal repeat providing sequences that can be converted into the mature right end of the next genome to be packaged. Gp18 and gp19 are required to create both the right end and, after packaging, the mature left end. However, the nuclease that recognizes the termini of mature T7 DNA in a concatemer is not yet known. During DNA packaging, the procapsid undergoes structural changes into an expanded icosahedral form. The scaffolding protein gp9 is thought to leave the procapsid at this stage. Once the DNA is completely encapsidated, the conical tail is attached to the portal structure. In contrast to many other phages, the T7 tail is assembled on the mature head and does not form an independent structure. Six tail fibers, each consisting of gp17 trimers, are then attached to the sides of the tail (Studier, 1972; Roeder and Sadowski, 1977). By 15 min post-infection at 30˚C, the first mature phage particle becomes detectable.

The process of cell lysis is poorly understood. Genes *3.5* and *17.5* are known to be required. Gp17.5 appears to be the holin and may disrupt the membrane to form cell ghosts, allowing access of gp3.5 amidase to the peptidoglycan where it can effect lysis (Young, 1992).

Abortive infection of T7 in F plasmid containing hosts

Bacteriophage T7 cannot undergo a productive infection in cells harboring the F plasmid (Makela *et al.*, 1964). T7 is therefore described as a " female-specific" phage and the failure to grow in F-containing (male) bacterial strains is now referred to as F exclusion. Early stages of the abortive infection are normal: the phages adsorb to male and female cells with equal efficiency, the first half of the T7 genome enters normally, the phage DNA is not degraded, and class I proteins are synthesized normally. However, the infection become grossly aberrant starting about 8 min after infection. Only half of the infecting phages complete genome translocation into male cells (Garcia and Molineux, 1995b), transcription rates of class II and III genes are rapidly reduced and synthesis of class II and III proteins is severely inhibited. Infected male cells also exhibit a loss of membrane integrity; as a result, acid-soluble phosphorus-containing compounds, most notably nucleoside triphosphates, and other small ions leak out of the abortively infected cell. Cells also fail to accumulate amino acids and become permeable to O-nitrophenyl-β-D-galactoside (Britton and Haselkorn, 1975a, b; Condit and Steitz,

1975; Condit, 1975). Host DNA is not completely degraded and no phage DNA synthesis is detectable. Finally the infected cells die but do not lyse. The numerous defects that accompany this abortive infection have made it difficult to separate the primary cause of phage exclusion from secondary or subsequent effects. Intensive studies have been conducted to elucidate the primary cause of the T7 abortive infection. However, there is still no agreement as to its cause.

Malamy and co-workers first reported that only class I proteins were detected during the abortive infection even though RNA isolated from both male and female T7 infected cells appeared identical (Morrison and Malamy, 1971). However, Whitaker *et al* (1975) noted that late T7 mRNAs were present, albeit in decreased amounts, in infected male cells. Beck and Molineux (1991) later confirmed that in infected male cells synthesis of late mRNAs was inhibited and that inhibition was more severe for RNAs transcribed from DNA near the right end of the genome. This observation became understandable when further evidence was provided showing that about half of infecting phages failed to translocate their entire genome into the cell (Garcia and Molineux, 1995b). In addition, by measuring the accumulation of early and late proteins, Yamada and Nakada (1975) and Blumberg *et al.* (1976) concluded that the translation machinery isolated from T7-infected male cells exhibited a decreased rate of protein synthesis. They postulated that the inhibition of translation led to the abortive infection of T7 in male host cells. However, others reported that no defect could be found in the

translational machinery isolated from infected male cells (Condit and Steitz, 1975; Ponta *et al*.,1975; Young and Menard, 1975).

Several investigators hypothesized that the change of membrane permeability is probably the cause of the abortive infection (Condit and Steitz, 1975; Britton and Haselkorn, 1975a). However, T7 mutants that grow normally in male hosts still cause nucleotide leakage from the infected cells (Molineux *et al*., 1989; Schmitt *et al*., 1991b), though perhaps at lower rates or only at later times than normal. Furthermore, chromosomal mutations that suppress F exclusion do not prevent leakiness (Remes and Elseviers, 1980; Schmitt *et al*., 1991b; Wang *et al*., 1999). Thus, membrane leakiness is a phenomenon that may be closely associated with F exclusion but it cannot be the primary cause.

F *pifA*

Morrison and Malamy (1971) defined the genes of F that cause T7 to abort an infection as *pif,* for phage inhibition factor. They concluded that *pif* included at least two genes, *pifA* and *pifB.* A new gene, *pifC,* was identified later and its product shown to negatively regulate the expression of the *pif* operon (Miller and Malamy, 1983). Expression of *pifC* from a plasmid does not prevent T7 growth but it does inhibit conjugal transfer of the broad-host-range plasmid RP4 (Cram *et al*., 1984; Miller and Malamy, 1983; Tanimoto and Iino, 1989).

PifA encodes an 80 kDa protein and mutations in *pifA* abolish F exclusion of T7 (Rotman *et al*., 1983). Further evidence showed that a plasmid expressing *pifA* as the only F gene inhibited T7 growth, suggesting that *pifA* is the only F gene responsible for F exclusion (Schmitt and Molineux, 1991). No biochemical function has been ascribed to PifA. The very existence of *pifB* was very controversial; it is now established that no such gene exists. DNA sequencing of the putative *pifB* region showed no open reading frame that could correspond to the hypothetical PifB. Mutations that were supposed to define *pifB* are likely to be leaky *pifA* mutations.

Phage mutants that grow in male cells

1. T3 mutants

T3 and T7 are closely related phages and exclude each other in co-infections. They have comparable patterns of RNA and protein synthesis, their genetic maps have been aligned (Beier and Hausmann, 1973) and the sequence of the T3 genome is now known to be about 75% identical to that of T7. However, T3 possesses a distinct property: an ability to grow normally in male strains. This interesting observation led to its employment as a model to investigate the mechanism of F-exclusion of T7. Early studies of a T3-T7 recombinant revealed two regions of T3 DNA that were necessary for the hybrid phage to grow in male strains (Molineux *et al*., 1983; Spence *et al*., 1983). One of these regions included

T3 genes *1.1* and *1.2*, which were shown to function in *trans* and complement the defects that caused T7 to be excluded by F (Spence *et al.*, 1983). Mutants of T3, altered only in gene *1.2*, were found to be restricted on male cells and behave like wild type T7 (Spence and Molineux, 1984). These observations indicate that T3 gene *1.2* is important in suppressing the inhibition by F and, conversely, that T7 gene *1.2* is defective in this activity.

Pseudorevertants of a T3 gene *1.2* deletion mutant able to plate efficiently on male cells were subsequently isolated. All pseudorevertants contain at least two mutations in gene *10* (encoding the major capsid protein), suggesting that gene *10* is responsible for F exclusion of a T3 gene *1.2* mutant. It was suggested that the mutations in gene *10* caused a reduction in the rate of gp10 synthesis, and thus a reduction in toxicity to the infected male cell (Condreay and Molineux, 1989). Evidence was also provided that wild type capsid protein (gp10) exerted its toxicity without being assembled into procapsids. The idea developed that expression of gene *10* was toxic to cells containing F and that the function of T3 gene *1.2* was to alleviate toxicity and allow phage growth.

2. T7 mutants

The successful characterization of T3 gene *1.2* pseudorevertants that are resistant to F exclusion led to the construction of T7 mutants able to overcome exclusion by F (Molineux *et al.*, 1989). These T7 mutants necessarily contain either a missense or null mutation in gene *1.2* and at least two missense mutations

in gene *10*. Expression of the wild type allele of either gene *1.2* or *10* results in T7 exclusion by F. The double null mutant of gene *1.2* and *10* does not show any of the physiological defects associated with the abortive infection (Beck and Molineux, 1991). Thus the products of gene *1.2* and gene *10* are the targets for *pifA*. It is not known how gp10 and gp1.2 exhibit their toxicity on *pifA*$^{+}$ cells or how T7 mutants escape F exclusion. However, mutations in T7 gene *10* are believed to manifest their effects through the same mechanisms as those in T3 (Condreay *et al*., 1989; Molineux *et al*., 1989). The mutations in genes *1.2* and *10* may reduce the toxicity of gp1.2 and gp10A by altering their interaction with either PifA or a cellular function.

Plasmids expressing wild-type T7 gene *1.2* or gene *10* of either T3 or T7 transform male strains at very low efficiency, relative to the isogenic female strains (Schmitt and Molineux, 1991). In the presence of *pifA*, controlled expression of either wild-type T7 gene *1.2* or gene *10* from plasmids result in the same physiological dysfunctions associated with the abortive phage infection. Plasmids containing missense alleles of T7 gene *1.2*, obtained from phages that grow in the presence of F, transform male cells with moderate to high efficiency (Schmitt and Molineux, 1991). Furthermore, overexpression of the same mutant T7 gene *1.2* from a high copy plasmid allows wild-type T7 to grow in male cells. Thus high levels of mutant T7 gp1.2 in the cell prior to infection prevent not only phage-encoded wild-type gp1.2 but also gp10 from interacting with PifA. However,

mutant gene *1.2* does not provide this activity when expressed from the phage genome (Molineux *et al*., 1989). In contrast, the presence of a single copy of T3 gene *1.2* in a T7 genome is sufficient to allow escape from F exclusion (Garcia and Molineux, 1995a). These observations suggest that T3 gp1.2 and mutant T7 gp1.2 may work similarly to prevent wild-type T7 gp1.2, and also gp10, from triggering the abortive infection, except that T3 gp1.2 is more efficient.

Phage genes involved in F exclusion

1. Gene *1.2*

Gen*e 1.2* is located near the primary replication origin of T7 or T3 (Saito *et al*., 1980; Dunn and Studier, 1983). It is transcribed early by *E. coli* RNA polymerase and late by the phage RNA polymerase. The ribosome binding site of T7 (but not T3) gene *1.2* is normally embedded in a hairpin structure within the gen*e 1.1* coding sequence and translation of gene *1.2* is dependent on translation of gene *1.1* (Saito and Richardson, 1981).

In addition to its role in F exclusion, a distinct function of gene *1.2* is known. Gene *1.2* is essential for T7 DNA replication in *E. coli* strains carrying the *optA1* mutation, which causes the overproduction of cellular dGTP triphosphohydrolase (dGTPase) (Beauchamp and Richardson, 1988). Gp1.2 has been shown to specifically inhibit *E. coli* dGTPase by a reversible association with an apparent stoichiometry of two gp1.2 monomers to one dGTPase tetramer

(Huber *et al*., 1988). T3 gp1.2 has also been shown to inhibit *E. coli* dGTPase. T7 *1.2* mutants are unable to grow on *optA1* strains due to the decreased pool of intracellular dGTP caused by the high levels of dGTPase. The primary sequences of T7 and T3 gp1.2 share 40% identity.

2. Gene *10*

Gene *10* of T7 and T3 codes for two proteins, gp10A and gp10B. Gp10B contains a C-terminal extension that is produced by a shift to the -1 reading frame near the normal gene *10A* termination codon (Dunn and Studier, 1983; Condreay *et al*., 1989; Condron *et al*., 1991). Gp10A is the major capsid component even though both gp10A and gp10B are assembled into wild type phage particles. Either protein alone is sufficient for viability. The primary sequences of T3 and T7 gp10A are 79% identical, but there is no similarity in the C-terminal extensions of gp10B (Condreay *et al*., 1989). Distinct from its structural role, synthesis of either gp10A or gp10B in male cells triggers the abortive infection (Molineux *et al*., 1989; Schmitt and Molineux, 1991).

Host mutations that affect F exclusion

FxsAp109

An *E. coli* mutant was isolated by selecting survivors of co-expressed gene *10A* and *pifA* (Wang *et al*., 1999a). The mutant allows T7 to plate at normal efficiency in the presence of F; the strain contains a promoter-up mutation in *fxsA (fxsAp109*)

that causes 25-fold overexpression of *fxsA*. Increased synthesis of FxsA, a cytoplasmic membrane protein, alleviates the inhibition of T7 growth by F (Wang *et al.*,1999b). Defects in both late phage protein synthesis and the host membrane integrity persist at reduced levels, but T7 grows moderately well in *fxsAp109* mutants that contain F. Disruption of $fxsA^+$ does not abolish the toxicity of the combination of PifA and either T7 gp1.2 or gp10, and it was suggested that FxsA plays a protective role, rather than being directly involved in the biochemical pathway of F exclusion.

Several other mutants of *E. coli*, originally isolated for other purposes, have also been found to affect the F mediated abortive infection of T7. In general, the mutations in these strains confer pleiotropic phenotypes and many of them change the severity of F exclusion of T7 simply by altering the level of PifA expression. Unfortunately, these mutants have not helped to illuminate the mechanism of T7 exclusion by F. The genes affected include *strA (rpsL)* (ribosomal protein S12), *galU* (UTP α-D-glucose-1-phosphate uridyltransferase) *rho* (transcription termination factor), *himA* (integration host factor IHF), *gyrA* (DNA gyrase), and *rpoB* (RNA polymerase β subunit).

strA and rpoB

Chakrabarti and Gorini (1975) reported that specific mutations conferring streptomycin resistance (Sm^R) in an Hfr strain of *E. coli* increase the plating efficiency of T7 by several orders of magnitude. Some Sm^R derivatives are

completely permissive for T7, others plate T7 at reduced efficiencies and yield small plaques, and yet others remain fully restrictive. Subsequently, the same authors made the even more interesting observation that the effects of these *strA* mutations on F exclusion could be reversed by mutations (*rpoB*) that confer resistance to rifampin (Chakrabarti and Gorini, 1975). However, these *rpoB* mutations have no effect on phage growth in the absence of the *strA* mutation.

Schmitt *et al.* (1995) showed that the mutations in *strA* that allow T7 to grow in strains containing F reduce the level of expression of a *pifA-lacZ* fusion, whereas those *rpoB* mutations that reversed the effect of the *strA* mutations increased expression. This suggests that the *rpsL* and *rpoB* mutations exert their effects on F exclusion of T7 merely by controlling synthesis of PifA. The *rpsL* mutations that alleviate F exclusion were also shown to slow the speed of ribosome movement (Galas and Branscomb, 1976; Zengel *et al*., 1977). It is also believed that a slower rate of translation might uncouple the ribosome from RNA polymerase and allow an increased frequency of Rho-dependent transcriptional termination (Imamoto *et al*., 1970) or allow increased nucleolytic degradation of mRNA not protected by ribosomes (Rosenberg and Schlessinger, 1982). Either process could also result in lower level expression of *pifA*.

Gyr, rho, himA and galU

Male strains containing either the *rho-702*(Ts) or *gyrA43*(Ts) mutation were found to allow moderate T7 growth (Schmitt *et al*., 1995). At permissive

temperatures for the Ts mutation the *gyrA43* mutation increases the plating efficiency of T7 in male cells, relative to the *gyrA*$^{+}$ male parent, by at least seven orders of magnitude. In addition plaques are almost normal. At 37°C β–galactosidase activity produced from the *pifA-lacZ* fusion plasmid is substantially reduced by the *gyrA43* mutation in both exponential and stationary phases of growth. Therefore, male cells containing the *gyrA43* mutation are likely to be phenotypically PifA^{-}. However, the mechanism by which the *gyrA43* mutation affects expression of *pifA* remains unknown.

E. coli rho mutants have extremely pleiotropic phenotypes. *rho* mutations affect transcription termination, recombination, sensitivity to UV irradiation, and DNA superhelicity (Arnold and Tessman, 1988; Arnold *et al*., 1989; Guarente and Beckwith, 1978; Das *et al*., 1976). At both 30° and 37°C, the *rho-702* mutation increases the plating efficiency of T7 in male cells by at least 4 orders of magnitude (Schmitt *et al*., 1995). The plating efficiency of T7 in male cells containing the *rho-702* mutation is improved by at least 7 orders of magnitude at 43°C, a temperature at which the host is still viable but contains reduced Rho activity. β-Galactosidase activities produced from a *pifA-lacZ* fusion plasmid in *rho-702* cells are similar to those produced in *rho*$^{+}$ cells. This result indicates that a reduction of PifA synthesis is not a major reason for the alleviation of F exclusion in *rho* mutants. However, it was suggested that the *rho-702* mutation might alter expression of an *E. coli* protein that is more directly involved in the biochemistry

of F exclusion because normal development of T7 does not require Rho (Schmitt *et al*., 1995).

Kennedy *et al.* (1988) reported that mutations (*himA* or *himD*) in IHF increased the plating efficiency of T7 on male strains. Construction of a *pifC-galK* promoter fusion to measure galactokinase levels in IHF mutant strains showed that promoter activity decreased about two-fold. It was proposed that the absence of IHF alters DNA conformation such that not only is the activity of the *pif* promoter reduced but also repression by PifC at *pifO* is enhanced.

Mutations in *galU* were also shown to decrease the inhibition of T7 by F (Remes and Elseviers, 1980) but the mechanism of suppression is not understood. It was suggested that the lack of UTP α-D-glucose-1-phosphate uridyltransferase causes an altered lipopolysaccharide that in turn might cause T7 to enter the host through altered receptors. It was further suggested that F-mediated inhibition might depend on the attachment and entry process.

Unfortunately none of the proteins encoded by these genes directly participates in the F exclusion pathway. Although FxsA protects T7 from exclusion by F and the mutant containing *fxsAp109* was obtained for the purpose of finding cellular components directly affected by the F exclusion system, it does not play a direct role. Gp1.2, gp10 and PifA are the only known proteins that are directly involved in F exclusion.

In this study both genetic and biochemical approaches were employed in the hope of identifying the cellular target of the F protein PifA, T7 gp1.2 and gp10A, and of obtaining direct evidence for interactions among those toxic proteins. The data presented below provide us with a better understanding of the molecular basis underlying F exclusion of T7.

Chapter 2. Materials and Methods

***E. coli* strains, phage strains and plasmids**. These are listed in Tables 1and 2.

Media and antibiotics

L-broth was usually used for bacterial growth, each liter of L-broth contains 10g Bacto-Tryptone, 5g Bacto-Yeast extract, 5g NaCl (Miller, 1972). Phage titering usually employed T broth. T broth contains 10g Bacto-Tryptone and 5g NaCl per liter. Plates were made with broth containing 1.5% Bacto-agar; 0.7% Bacto-agar dissolved in T broth was used to make T-soft agar. MacConkey plates were made with Bacto-MacConkey base agar supplemented with 1% sugar. One liter of M9 minimal medium is composed of 1g NH_4Cl, 1mM $MgSO_4$, 3g KH_2PO_4, 6g NaH_2PO_4 and 2g sugar, usually glucose (Studier, 1969). The following antibiotics were used: ampicillin (100 µg/ml), kanamycin (25 µg/ml), tetracycline (20 µg/ml) and chloramphenicol (40 µg/ml).

Enzymes

Restriction endonucleases, T4 DNA ligase, the large fragment (Klenow) of *E. coli* DNA polymerase I, and T4 DNA polymerase were from New England Biolabs. Calf intestinal alkaline phosphatase (AP) and Proteinase K were from Boehringer Mannheim. Taq DNA polymerase was a gift from Dr. J. J. Bull.

Growth of T7

Mid-log cells of a permissive strain were infected with a small stock of

Table 1. Bacterial and phage strains used in this work

Strains	Genotype	Sources of references
BL21(DE3)	F⁻ *omp*T *gal dcm lon* hsdS_B (λD69 int: T7 gene *1*)	Studier *et al*., 1990
CAG18493	MG1655 *zbi-29*::Tn (17.7')	Nichols *et al.*, 1998
CC118	*araD139* Δ*(ara-leu)7697* Δ *lacX74*Δ *phoA20* *galE galK thi rpsE*(Spc^R) *rpoB*(Rif^R) *argE*(Am) *recA1*	Manoil& Beckwith,1985
IJ511	Δ *lacX74 galK2 galT22 supE44 hsdS*	Condreay & Molineux,1989
IJ512	IJ511 F'*lac* (F42)	
IJ1023	IJ1198 *fxsA*::Kn	Wang *et al*., 1999
IJ1029	IJ1198 *fxsA*::Cm	Wang *et al*., 1999
IJ1198	Δ *lacX74 galK150*(Am) *cys*(Am) *Trp*(Am) *supF*	Wang *et al*., 1999
IJ1199	IJ1198 *fxsAp109*	Wang *et al*., 1999
IJ1200	IJ1198 Δ *(srlR-recA)306::*Tn10	Wang *et al*., 1999
IJ1291	IJ1199 *fxsA*::Cm	Wang *et al*., 1999
IJ1818	IJ511 *pnp::Tn5*	This work
IJ1819	IJ512 *pnp::Tn5*	This work
IJ1820	IJ1198 T5^R	This work
IJ1821	IJ1199 Nal^R	This work
TB1	*ara* Δ(*pro-lac*) *strA thi* (Φ80d *lacZ*Δ*M15*) *hsd*R	Yanisch-Perron, *et al*., 1985
Top10	F⁻ *mcr*A Δ(*mrr-hsdRMS-mcrBC*) [Φ80d *LacZ*Δ*M15*] Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (Str^R) *endA1 nupG*	Invitrogen Co.
Bacteriopages		
T3		
Δ 2	T3 Δ (*1.05-1.2*)	Schmitt et al., 1987
T7		
T7$^+$	Wild type (F. W. Studier)	
ST16	Deletion of T7 genes *1.1* and *1.2*	Meyer *et al*., 1987
MM10	*10A2D 10A228G*	Molineux *et al*., 1989
MM20	*1.2 W31C 10A2D 10A228G*	Molineux *et al*., 1989
λ		

λ1.2pifK	λgt11S$^+$, T7 gene *1.2* and *pifA*(Am), *c*I857, Kn^R under *lac* promoter control.	Wang *et al*., 1999
λ10pifK	λgt11S$^+$, T7 gene *10* and *pifA*(Am) *c*I857, Kn^R under *lac* promoter control	Wang *et al*., 1999
434-1.2pifA	Recombinant between *imm*434 *c*Its56*plac5* and λ1.2pifK	Wang *et al*., 1999
434-10pifA	Recombinant between *imm*434 *c*Its56*plac5* and λ10pifK	Wang *et al*., 1999

Table 2. Plasmids used in this work

Plasmids	Features	Source or reference
T7 gene *1.2* plasmids		
pAK25	pUC9; T7 gene *1.2* under P*lac* control.	Schmitt, *et al.*, 1991
pCS5	pUC19; T7 gene *1.2 W31C* under P*lac* cont	Garcia *et al*., 1995
pGST1.2	pGEX-3X, *gst*-T7 *1.2* under P*lac* cont	This work
pGX100	pET-11d, T7 *1.2* under T7 promoter control.	This work
pGX105	pHE11; T7 *1.2Y28N* under T7 promoter control	This work
pGX106	pHE11; T7 gene *1.2W31C* under T7 promoter	This work
pGX107	pHE11; T7 gene *1.2* under T7 promoter control.	This work
pGX109	pHE11; T7 gene *1.2R37G* under T7 promoter control.	This work
pHis1.2	pTrchisA; his-tagged T7 *1.2* under P*trs* control.	This work
T7 gene *10* plasmids		
pET10	pET-11d; T7 *10A*$^+$ under T7 promoter control.	This work
pET10PC	pET11d; T7 *10A A2D,F291S(Ts)* under T7 promoter control.	This work
pET10JT	pET11d; T7 *10A A2D,A228G(Ts)* under T7 promoter control.	This work
pHis10A	pTrchisB; his-tagged T710A under P*trc* control.	This work
pPK13	pUC19, T7 *10A* under P*lac*.	Lab collection
pWSK10A	pWSK29, T7 *10A* under P*lac*.	This work
***pifA* plasmids**		
pGX25	pTrchisB; truncated *pifA* (del 1-462) expressing C-terminal 254 codons.	This work
pGX26	pTrchisB; truncated *pifA* (del 1-462) expressing.	This work

	C-terminal 536 codons	
pGX31	pTrchisA; *pifA K59A* under P*trc* control.	This work
pHispifA	pTrchisA; his-PifA under P*trc* control.	This work
pPK5	pKS$^+$; *pifC* and *pifA* under P*lac*.	Lab collection

***fxsA* plasmids**

pGSTFxsA-C	pGEX-3X; truncated *fxsA* expressing C-terminal 37 codons fused to *gst*.	This work
pHisFxsA	pTrchisB; his-FxsA under P*trc* control.	This work
pHisFxsA102	pTrchisA; N-terminal 102 residues of FxsA.	This work
pHisFxsA107	pTrchisA; N-terminal 107 residues of FxsA.	This work
pHisFxsA111	pTrchisA; N-terminal 111 residues of FxsA.	This work
pHisFxsA115	pTrchisA; N-terminal 115 residues of FxsA.	This work
pHisFxsA121	pTrchisA; N-terminal 121 residues of FxsA.	This work
pWF184	pWSK29, 725bp TaqI of *fxsAp109* under P*lac*.	Wang *et al.*, 1999
pWF185	pWSK29, 725bp TaqI of *fxsA*$^+$ under P*lac*.	Wang *et al.*, 1999
pFxsA18	pSWFII, C-terminal deletion FxsA fused to PhoA at codon S18, under P*fxsA*$^+$	Wang, 1998
pFxsA24	pSWFII, N-terminal FxsA fused to PhoA at codon A24, under P*fxsA*$^+$	Wang,1998
pFxsA32	pSWFII, N-terminal FxsA fused to PhoA at codon T32, under P*fxsA*$^+$	Wang,1998
pFxsA40	pSWFII, N-terminal FxsA fused to PhoA at codon S40, under P*fxsA*$^+$	Wang,1998
pFxsA45	pSWFII, N-terminal FxsA fused to PhoA at codon S45, under P*fxsA*$^+$	Wang,1998
pFxsA47	pSWFII, N-terminal FxsA fused to PhoA at codon V47, under P*fxsA*$^+$	This work
pFxsA53	pSWFII, N-terminal FxsA fused to PhoA at codon T53, under P*fxsA*$^+$	Wang,1998
pFxsA68	pSWFII, N-terminal FxsA fused to PhoA at codon P68, under P*fxsA*$^+$	Wang,1998
pFxsA77	pSWFII, N-terminal FxsA fused to PhoA at codon S77, under P*fxsA*$^+$	This work
pFxsA84	pSWFII, N-terminal FxsA fused to PhoA at codon L84, under P*fxsA*$^+$	This work
pFxsA88	pSWFII, N-terminal FxsA fused to PhoA at codon P88, under P*fxsA*$^+$	Wang, 1998

pFxsA95	pSWFII, N-terminal FxsA fused to PhoA at codon L95, under P*fxsA*$^{+}$	This work
pFxsA102	pSWFII, N-terminal FxsA fused to PhoA at codon P102, under P*fxsA*$^{+}$	This work
pFxsA122	pSWFII, N-terminal FxsA fused to PhoA at codon P122, under P*fxsA*$^{+}$	This work
miscellaneous		
pBH176	pBR322; expressing his-tagged SP6 RNA polymerase of SP6 under T7 promoter control.	McAllister *et al*.,
pET-11d	pBR322; T7 promoter expression vector.	Studier *et al*., 1990
pGEX-3X	pBR322; GST fusion vector; *tac* promoter.	Pharmacia Biotech
pHE11h	pET11-c; N-terminal his-tag vector under T7 promoter control.	Harshey lab
pLys(S)	pACYC184; synthesizing T7 lysozyme.	Studier *et al*., 1990
pPK41	pACYC184, T7 RNA polymerase gene *1*.	Lab collection
pQ	pACYC184::*lacI*^{Q}*Tc*^{R}	Lab collection
pSWFII	pBR322, signal-less & promoter-less *phoA.*	Ehrmann *et al*., 1990
pTrchisA,B,C	pBR322, N-terminal his-tag vector, under P*trc.*	Invitrogen
pTrcPNP	pTrc99A, synthesizing C-terminal his-PNPase under P*trc*	This work
pTrc99A	pBR322, C-terminal his-tag vector, under P*trc.*	Pharmacia Biotech
pWSK29	pSC101 replicon, cloning vector.	Wang *et al*., 1991

phage and the culture was incubated aerobically at 30˚C until lysis. The lysate was made 1M NaCl by the addition of solid NaCl and cell debris was removed by centrifugation at 10,000 rpm for 15 min; phage stocks were stored at 4˚C. When purified phage was required, the lysate was made 6% polyethylene glycol 8000 and stored at 4˚C overnight. Phages were collected by centrifugation at 5000 rpm for 10 min. After resuspension in T7 buffer (10 mM Tris-HCl, pH 8.0, 0.1mM EDTA, 1M NaCl) and a brief centrifugation, phages were loaded onto a 3-layer step gradient of CsCl (33g, 41g, and 50g per 50 ml H_2O; ρ = 1.35, 1.45, and 1.55) and centrifuged at 35K in a Beckman SW55 rotor for 2 hr. Phages were collected through the side of the tube with a syringe, dialyzed against T7 buffer, and stored at 4˚C.

Preparation of plasmid DNA

Plasmid DNA was prepared by an alkaline lysis procedure adapted from the protocol in Molecular Cloning (Maniatis *et al.,* 1989). An overnight culture (1.5 ml) of cells carrying the plasmid was centrifuged and suspended in 0.1 ml solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0). Two volumes of solution II (0.2M NaOH, 1% SDS) were added. After incubation for 5 min, the cell lysate was neutralized by addition of 1.5 volumes of solution III (3M NaOAc, pH 4.8) and incubated on ice for 5 min. After removal of cell debris by centrifugation, the supernatant was precipitated with 2 volumes of 95% ethanol. Plasmid DNA was dissolved in either H_2O or TE buffer (10 mM Tris-HCl, 1mM

EDTA, pH 8.0), treated with RNase (100µg/ml) and extracted with phenol/chloroform/isoamyl alcohol (25:24:1). After a brief centrifugation, the aqueous layer was precipitated with 2 volumes of 95% ethanol. Plasmid DNA was washed with 70% ethanol, dried and resuspended in TE or in H_2O. The concentration of DNA was determined by measuring A_{260} using the formula: [DNA] = A_{260} x 50 µg/ml.

Plasmid construction

Where PCR was part of a construction, the relevant parts of the final plasmid were sequenced to confirm their structure.

1. **pGX100**: T7 gene *1.2* was amplified by PCR using primers *Nco* I-1.2T7-6137F (5´–AGCTCCATGGGACGTTTATATAGTGG) and T7 6397R (5´–GCGGATCCACGTATTACTTCCAGTCC). The amplified gene is flanked by *Nco* I and *BamH* I sites. After digestion with *Nco* I and *BamH* I, gene *1.2* was cloned into pET-11d (*Nco* I/*BamH* I). This yielded pGX100 in which gene *1.2* is under T7 promoter control.

2. **pGST1.2**: pGEX-3X, a glutathione S-transferase (GST) fusion vector, was used as a backbone to construct pGST1.2. pGEX-3X is a pBR322 derivative and contains the coding sequence for GST protein (26 kDa) under the control of the IPTG-inducible *tac* promoter. T7 *1.2* gene was amplified by PCR using T7 DNA as a template. Primers 1.2T7 F (5´–ATGCAGATCTGGGTCAGTAAGATGGG) and T7 1.2-6397Ra (5´–GCGGATCCACGTATTACTTCCAGTCC) were

designed such that the amplified gene *1.2* was flanked by *Bgl* II and *BamH* I restriction sites. After digestion with *BamH* I and *Bgl* II, the amplified *1.2* gene was cloned into the *BamH* I site of the polylinker of pGEX-3X. This produced the plasmid pGST1.2 with gene *1.2* fused, in-frame, to the 3´ of *gst*.

3. **pGX107**: T7 gene *1.2* was amplified by PCR from T7 DNA using the primers, Nco I-1.2T7-6137F (5´–AGCTCCATGGGACGTTTATATAGTGG) and *BamH* I-1.2T7-6397Ra (5´–GCGGATCCACGTATTACTTCCAGTCC). The amplified gene *1.2* was flanked by *Nco* I and *BamH* I restriction sites. After gel purification and cleavage with *Nco* I and *BamH* I, gene *1.2* was cloned into pHE11h, previously digested with the same enzymes. The resulting plasmid pGX107 contained gene *1.2* under T7 promoter control, allowing inducible synthesis of a gp1.2 derivative with a N-terminal hexahistidine tag in *E. coli* strain BL21(DE3). Plasmids (pGX105, pGX106 and pGX109) expressing his-tagged T7 mutant gp1.2 were constructed by the same procedure.

4. **pET10A**: T7 gene *10A* was excised from pPK13 with *Nhe* I-*BamH* I and inserted into the polylinker of pET-11d cut with same enzymes creating pET10A. Expression of gene *10A* is under T7 promoter control and inducible with IPTG.

5. **pHis10A**: pPK13 containing T7 gene *10A* was cut with *Nde* I, made blunt-ended with Klenow fragment and digested with *Eco*R I to yield a *Nde* I (fill-in)-*Eco* RI gene *10A* fragment. The fragment was cloned into pTrchisB (N-terminal his-tag vector) digested with *Bgl* II (Klenow fill-in) and *Eco* RI to yield pHis10A from

which the N-terminal his-tagged *10A* gene was expressed under *trc* promoter control.

6. **pHisPifA**: a 2.4 Kb *Bgl* II DNA fragment containing the complete *pifA* gene was excised from pPK5 and inserted into the *Bgl* II site of pTrchisA, a N-terminal six histidine-tag vector. The resulting plasmid (pHisPifA) expresses a tagged PifA under the control of the *trc* promoter.

7. **pHisFxsA**: A Hinc II fragment containing *fxsA* from pWF185 was ligated into the *Bgl* II site (blunt ended by a fill-in reaction with Klenow fragment) of pTrchisA. Expression of *fxsA* is under *trc* promoter control.

8. **pGSTFxsA-C**: A DNA fragment (*Hinc* II-*Sph* I of *fxsA*, 111 bp) was excised from pWF185 and inserted into *Sma* I site of pGEX-3X. The construct expresses the last 37 amino acids of FxsA (residues 122-158) fused in frame to the C-terminus of GST.

9. **FxsA-phoA** plasmids: All fusions were constructed by ligating PCR products of various N-terminal coding sequences of *fxsA* into the promoter-less, signal sequence-less *phoA* plasmid pSWFII at the unique *Sma* I site (Ehrmann *et al*., 1990). Transcription of the fusion gene is from the $fxsA^+$ promoter. pSWFII is a pBR322-based replicon of medium copy number.

10. **pTrcPNP** plasmid: The gene encoding *E. coli* polynucleotide phosphorylase (PNPase) was amplified by PCR from genomic DNA of the strain MG1655. Primers PNPNcoI (5’-GCACCATGGCTATGCGCAGAAGATCG) and

PNPBamH (5'- GCGGATCCTTACTCGCCCTGTTCAGC) are based on the *pnp* sequence and the amplified gene is flanked by *Nco* I and *BamH* I sites. After amplification and cleavage with *Nco* I and *BamH* I, PCR products were cloned into pTRC99A digested with *Nco* I and *BamH* I. The resulting plasmid pTrcPNP expresses PNPase under *trc* promoter control with a C-terminal his-tag.

Selection for *E. coli* mutants tolerant to coexpression of *pifA* and gene *1.2* or *10*

λ1.2pifK and λ10pifK were used as a primary selecting phages. They are derivatives of λgt11 lacking the *Sam100* mutation. λ1.2pifK contains T7 gene *1.2*, *pifA* and Kn^R; λ10pifK contains T7 gene *10*, *pifA* and Kn^R. These genes are co-expressed under *lac* promoter control. The selection for *E. coli* mutants that survive a challenge from T7 gene *1.2* or T7 gene *10* and *pifA* was conducted as follows. About $2x10^9$ stationary phase IJ1198 cells from each of 5 independent cultures were treated with nitrosoguanidine (50 µg/ml, 37°C for 10 min) to give 30% survival; following outgrowth cells were infected at room temperature with λ1.2pifK or λ10pifK at a multiplicity of 10. After removal of unadsorbed phage, infected cells were suspended in M9 galactose medium and incubated for 60 min at 30˚C. Cells were then plated on M9 galactose agar containing 25 µg/ml kanamycin. Cell growth thus requires maintenance of *supF* for suppression of the three amber mutations (*galK, trp, cysI*) in IJ11998 and the presence of λ1.2pifK or λ10pifK to provide Kn^r. Colonies were purified on the same media and patch-

tested at 30˚C on plates containing $5x10^7$ 434-1.2pifK or 434-10pifK (30˚C is non-permissive for the 434*c*I56(Ts) mutation) to screen for non-toxic prophages. 434-1.2pifK and 434-10pifK are the equivalent phages of λ1.2pifK and λ10pifK except that the immunity region *imm*λ is substituted with *imm434*. Only lysogens that harbor a mutation conferring tolerance to gene *1.2* and *pifA* coexpression (or gene *10* and *pifA*) allow lytic growth of 434-1.2pifK (or 434-10pifK). Sufficient numbers of cells were applied to each patch such that clearing due to phage growth would be obvious and that confluent growth resulted if the strain could not propagate the tester phage. Mutants were then tested for their ability to support the growth of a T4 *55*(Am) mutant to ensure that *supF* activity was not reduced.

Cell growth and preparation of cell extracts

For genetic overexpression and purification of proteins, cells were normally grown at 37˚C to A_{600} of 0.7-0.8 and induced with 1mM IPTG for 2-3 hr. Cells were harvested by centrifugation at 5000 rpm for 10 min at 4˚C, resuspended in either PBS (140 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, KH_2PO_4, pH 7.4) for GST fusion proteins, or 50 mM NaH_2PO_4 pH 8, 300 mM NaCl and 10mM imidazole for his-tagged proteins (50 µl per ml of original cell culture), and were then disrupted by passage through a French pressure cell at 16,000 p.s.i. The cell lysate was made 10 mM $MgSO_4$ and incubated with 1% Triton X-100 (his-tagged and GST fusion proteins only), 5 µg/ml DNase and 1mM PMSF at room

temperature for 10 to 20 min with occasional mixing. Cellular debris was then removed by centrifugation at 10,000 rpm for 10 min at 4˚C.

FxsA was prepared from IJ1200/pWF185. Cells were grown at 37 °C to A_{600} =1.5, harvested by centrifugation and resuspended in a solution (50 mM NaH_2PO_4 pH 8.0, 25 mM NaCl, 10 mM imidazole, 50 µl per ml of original culture). Cell extracts were made as described above except that Triton X-100 was added to 0.5% in order to enhance solubilization of FxsA from the membrane.

To prepare wild-type and mutant T7 gp10A, 25 ml of BL21DE3(pLysS) containing pET10A, p10A-PC10 or p10A-JT4 were grown to A_{600} = 0.5 and induced with 1mM IPTG at 30˚C or 37˚C for 2 hr. Cells were resuspended in 5 ml PBS. Cell extracts were prepared as described above.

Preparation of cytoplasmic and membrane fractions for cellular localization of proteins

1. Cellular localization of PifA and N-terminal truncated PifA

A 200 ml culture of IJ511/F´*lac* at A_{600} = 0.7 was harvested by centrifugation. Cells were resuspended in 5 ml PBS (pH 7.4) and broken by passage through a French pressure cell. The total membrane fraction was collected by centrifugation at 100,000 x g for 2 hr at 4˚C and resuspended in 5 ml PBS (pH 7.4). For the Sarkosyl solubility assay, the total membrane suspension was incubated on ice with 0.5% Sarkosyl for 10 min and centrifuged at 100,000 x g for 2 hr at 4˚C. After dissolving the pellet in 5 ml PBS, both the pellet suspension and

the supernatant were dialyzed against PBS (pH 7.4). In some experiments, the membrane pellet was suspended in 1M NaCl. After incubation with shaking at 4˚C for 20 min, the membrane was collected as described above. Samples from each fraction were subjected to SDS-PAGE and examined for the presence of PifA by immunoblotting using anti-PifA antibody. N-terminal truncated PifA was localized by the same procedure as above except that Top10(pGX25) and Top10(pGX26) were the strains used.

2. Cellular localization of T7 gp1.2 and gp10A

A 100 ml culture of IJ1199(Q)(pAK25) at A_{600} = 0.4 was induced with 0.1mM IPTG until A_{600} = 0.7-0.8. IJ1199(Q)(pWSK10A) was grown to A_{600} = 0.7-0.8 at 37˚C without IPTG induction. Harvested cells were resuspended in 5 ml of 50 mM Tris-HCl pH 8.0 and disrupted using a French press. After removal of cell debris, cell extracts were centrifuged at 100,000 x g at 4˚C for 2 hr. The total membrane fraction was suspended in 50 mM Tris-HCl pH 8.0. The presence of gp10A and gp1.2 in the cytoplasmic and membrane fractions was determined by SDS-PAGE and immunoblotting using anti-10A or anti-1.2 antibody. Protein concentrations were measured by using the Bradford reagent (Bio Rad).

Preparation of inner and outer membranes

Membranes were prepared from strain IJ511/F'*lac* using the method of Osborne *et al.* (1972). A 200 ml culture was grown to a cell density of 5 x 10^8 per ml at 37˚C. Cells were harvested by centrifugation at 5000 rpm, resuspended in 10

ml of an ice cold solution of 0.75M sucrose, 10 mM Tris-HCl (pH 7.8) and incubated with 100 µg/ml lysozyme for 2 min. Twenty ml of ice-cold 1.5 mM EDTA (pH 7.5) were then added slowly with gentle mixing over a period of ten min. Spheroplasts were broken by one passage of the suspension through a French pressure cell at 16,000 p.s.i. After removal of cell debris by centrifugation at 10,000 rpm for 10 min, crude membranes were collected by centrifugation at 150,000 x g for 2 hr at 4˚C and gently resuspended in 10 ml of 0.25 M sucrose, 3.3 mM Tris-HCl, 1mM EDTA (pH 7.5). After centrifugation at 150,000 x g for two hr at 4˚C, the membrane fraction was resuspended in 1ml 25% (w/w) sucrose, 5mM EDTA (pH 7.5), and was then loaded on top of a step sucrose gradient (0.5 ml of 55%, 0.7 ml each of 50%, 45%, 40% 35%, and 30% sucrose (all w/w)). Inner and outer membranes were separated by centrifugation in a SW 55 rotor at 120,000 x g for 14 hr at 4˚C; 250 µl fractions were collected from the tube bottom. Protein concentration was determined using the Bradford reagent. For assaying the activity of NADH oxidase, 5 µl of each gradient fraction was incubated with 1ml of 50 mM Tris-HCl (pH 7.5), 0.12 mM NADH, 0.2 mM dithiothreitol, at 37˚C for 10 min. NADH oxidase activity was measured as the decrease in absorbance at 340nm. Relative amounts of PifA and OmpA in fractions were determined by a western blot using anti-PifA and anti-OmpA (a gift from Dr. C. Earhart) antibodies after electrophoresis through a 12% SDS-PAGE gel and electrotransfer of the proteins to a PVDF membrane.

SDS-PAGE and Immunoblotting

Proteins were resolved on 12% (gp10A, PifA and FxsA) or 15% (gp1.2) Tris-glycine SDS-PAGE gels. Proteins were then electrophoretically transferred to a PVDF membrane (Millipore Co.). After one hour of blocking with 5% nonfat milk in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5), the membrane was incubated with primary antibody in TTBS (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween 20, pH 7.5) for 1 hr and then incubated with a TTBS solution containing a secondary antibody conjugated with alkaline phosphatase for 1 hr. Primary antibodies were used at a dilution of 1:10,000 for anti-10A, 1: 5,000 for anti-PifA, and 1: 3,000 for anti-1.2 and FxsA. Secondary antibodies were diluted in TTBS at 1: 5,000 for goat anti-rabbit (Bio Rad) and 1:3,000 for goat anti-mouse (Kirkegard and Perry Lab, Inc). Protein bands of interest were visualized using AP color reagents from Bio Rad Laboratory.

Quantitative immunoblotting

To quantitate PifA in IJ511/F'*lac*, 100 ml of cells were grown at 30˚C to late log phase, harvested and resuspended in 2 ml PBS (pH 7.4). After disruption in the French press, the cell extract was subjected to SDS-PAGE. Various amounts of purified his-PifA were also loaded to the same gel to make a standard curve. After immunoblotting using anti-PifA serum, blots were scanned at 300 dpi and the digitized images were quantified with NIH Image software. For the quantification of gp1.2 and gp10A in F^- and F^+ strains after T7 infection, 300 ml of IJ511 or

IJ511/F'*lac* was infected with T7 at a m.o.i. of 0.4 or of 5 when the cell density reached $2x10^8$ /ml. Infected cells were harvested 20 min after infection and washed once with PBS (pH 7.4). After resuspension in 1.5 ml PBS, cells were disrupted in the French press. Cell extracts were subjected to SDS-PAGE followed by immunoblotting using anti-gp1.2 or anti-gp10A serum. Protein quantification was as for PifA except that purified gp1.2 or gp10A was used as a standard.

Antibody production

Polyclonal antisera were produced by following the procedure from "Current Protocols in Molecular Biology" (Struhl *et al.,* 1992). Briefly, purified protein in PBS (pH 7.4) was mixed and emulsified with an equal volume of complete Freunds adjuvant (CFA, Sigma). A dose corresponding to 0.5 mg protein was injected subcutaneously into a female New Zealand rabbit. Booster immunizations using incomplete Freunds adjuvant were administered three times every three weeks after the primary injection. The antisera were collected two weeks after each boost and stored in aliquots as crude serum at –20˚C.

Protein purification

1. **GST fusion proteins**:

Purification of GST fusion proteins was conducted following the batch purification protocol from Pharmacia Biotech. GST-1.2 and GSTFxsA-C were expressed from the strains Top10(pGST1.2) and Top10(pGSTFxsA-C). Cell extracts were absorbed to glutathione-Sepharose 4B beads (Sigma) (0.5ml beads

per 5 ml cell extract) for 2-4 hr at 4˚C with gentle agitation. The beads were gently pelleted and washed three times with 10 bed volumes of PBS. Bound GST fusion proteins were eluted with glutathione elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0), dialyzed against PBS and stored at –20˚C. The purity of GST fusion proteins was determined after electrophoresis using SDS-PAGE.

2. **His-tagged proteins**

His-tagged proteins were purified essentially by using Qiagen's protocol for batch purification. His-tagged proteins were expressed from the following strains: Top10(pHisPifA) for his-PifA, BL21(DE3)(pGX107) for his-1.2, BL21(DE3)(pGX105) for his-1.2 (Y28N), BL21(DE3)(pGX106) for his-1.2 (W31C), BL21(DE3)(pGX109) for his-1.2 (R37G), BL21(DE3)(pBH176) for SP6 his-RNA polymerase (RNAP), Top10(pHis10A) for his-10A and pTrcPNP for PNPase-His. A 50% Ni-NTA agarose slurry was added to cell extracts (1ml per 5 ml cell extract) and the mixture was gently shaken at 4˚C for 2-4 hr. The resin was collected and washed extensively with buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole). Bound protein was eluted with a step gradient of elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl) containing 40, 60, 80 and 250 mM imidazole. The his-tagged protein in each eluate was detected by SDS-PAGE, fractions containing the purest protein were pooled, dialyzed against 50 mM NaH_2PO_4 (pH 8.0), 50 mM NaCl, 10% glycerol and aliquots were stored at –20˚C.

3. **T7 gp1.2**

1.2 protein was purified essentially as described by Myers (1987).

Preparation of cell extract: Cells from 2 liters of an induced BL21(DE3)(pLysS)(pGX100) culture were suspended in 100 ml of buffer A (20 mM Tris-HCl, pH7.5, 10% glycerol, 2 mM β-mercaptoethanol, 50 mM NaCl, 0.1 mM EDTA) containing 1mM PMSF, 5 µg/ml DNase and 10mM $MgSO_4$ and were disrupted by passage through a French pressure cell. The supernatant, after centrifugation to remove unbroken and cellular debris, is Fraction I.

Streptomycin sulfate precipitation: Eleven ml of 40% freshly dissolved streptomycin sulfate was slowly added to 110 ml of Fraction I. The solution was stirred for 30 min and was then centrifuged at 1,600 x g for 30 min. Ammonium sulfate (38.5 g) was dissolved in 110 ml of the supernatant and the solution was stirred at 4˚C for 45 min. After centrifugation as above the precipitate was dissolved in Buffer A and dialyzed against the same buffer (Fraction II).

DEAE-Cellulose: Fraction II was loaded onto a 4.9 cm^2 x 21 cm column of DE32 DEAE-cellulose, equilibrated with buffer A. After washing with one volume of buffer A, the column was eluted using a one liter gradient of 50-500 mM NaCl in buffer A at a flow rate of 0.5 column volume per hr. Fractions containing gp1.2, as determined by SDS-PAGE gel analysis, were pooled, concentrated by precipitation with 80% ammonium sulfate, and were resuspended in 2 ml of buffer B (50 mM Tris-HCl, pH7.5, 10% (w/v) glycerol, 150 mM NaCl, 0.1 mM EDTA, 2 mM β-mercaptoethanol) (Fraction III).

Sephadex G-50: Fraction III was loaded onto a 25cm x 4.9cm^2 Sephadex G-50 column equilibrated with buffer B. The column was washed with buffer B at a flow rate of 12 ml per hour. Fractions containing gp1.2 were pooled as determined by SDS-PAGE gel analysis, concentrated by dialysis against solid polyethylene glycol 8000, and were dialyzed against buffer C (7.5 mM potassium phosphate, pH 7.4, 10% glycerol, 2 mM β-mercaptoethanol) (Fraction IV).

Hydroxylapatite: Fraction IV was loaded onto a 7cm x 1.8 cm^2 hydroxylapatite column equilibrated with buffer C. The column was washed with one column volume of buffer C and gp1.2 was then eluted with a gradient (100 ml) of 7.5-100 mM potassium phosphate (pH 7.4) in buffer C. Fractions containing gp1.2, as judged by SDS-PAGE, were pooled and dialyzed against buffer D (20 mM Tris-HCl pH7.5, 0.1mM EDTA, 50% glycerol). Purified gp1.2 was stored at –20˚C.

4. **T7 gp10A**

1). For antibody production.

T7 gp10A was purified by the method of Hager (1980). Purified T7 particles were electrophoresed on a gradient SDS-PAGE gel (7.5-17.5 %). The band of gp10A was visualized by staining with 0.25 M KCl and excised. Gel pieces were soaked in elution buffer (0.1% SDS, 50 mM Tri-HCl, pH 7.9, 0.1 mM EDTA, 0.15 M NaCl), were crushed with a Teflon pestle, and were agitated at 25˚C for 1 hr. After brief centrifugation, the supernatant was concentrated using an

Amicon Centurion 10 filter, dialyzed against PBS containing 0.05% NP-40, and stored at –20°C.

2). For protein interaction assays.

Cell extract: Gp10A was expressed from BL21(DE3)(pLysS)(pET10A) and cell extracts were made as described (Cerritelli *et al*., 1996). Cells were suspended in 1/5 of the original culture volume in buffer E (20 mM Tris-HCl pH 8.0, 3 mM β-mercaptoethanol, 10% glycerol, 2 mM EDTA, 100 mM NaCl) containing 1mM PMSF and 0.1% Triton X-100 and disrupted by passing through a French press. Extracts were then treated with DNase I (10 μg/ml) in the presence of 10 mM $MgSO_4$ for 15 min at room temperature and were then centrifuged at 5000 rpm for 10 min to remove cell debris.

Ammonium sulfate precipitation: A saturated ammonium sulfate solution was added to 200 ml cell extract to give a final concentration of 20%. The solution was stirred at 4˚C for 3 hr and then centrifuged at 3000 x g for 30 min. The precipitate was dissolved in buffer E and dialyzed against the same buffer overnight at 4˚C (Fraction I). Proteins in both the supernatant and precipitate were analyzed by SDS-PAGE.

DEAE-Sepharose: Fraction I was loaded onto a DEAE-Sepharose CL-6B column (5cm x $5cm^2$) previously equilibrated with buffer E. As gp10A does not bind to DEAE-Sepharose, the flow-through was collected and analyzed by SDS-PAGE. Fractions containing the purest gp10A were pooled and stored at –20˚C.

Protein interaction assays

1. Affinity chromatography

1). Batch affinity chromatography: 0.5 mg purified his-tagged protein in 0.5 ml of buffer F (50 mM NaH_2PO_4 pH8.0, 25 mM NaCl) was incubated with 0.1ml of a 50% slurry of Ni-NTA agarose beads equilibrated with buffer F. After gentle shaking for 2 hr at 4˚C the resin was washed three times with 1.5 ml of buffer F containing 10 mM imidazole. Centrifugation was used to remove unbound protein. An additional 0.5 mg of his-tagged protein was loaded onto the beads, incubated and washed as above. The concentration of bound protein was estimated by comparing the amounts of starting material and unbound protein. Beads were usually saturated with the his-tagged proteins used after the second loading; 0.2-0.3 mg of protein usually bound to the resin.

Ten µg of T7 gp1.2 or gp10A (in 0.5 ml) or 5 ml of a cell extract containing gp10A, gp1.2 or FxsA in buffer F plus 10 mM imidazole was incubated with 50 µl (drained volume) of Ni-NTA agarose beads bound with his-PifA or SP6 his-RNA polymerase (his-RNAP). After two hours of gentle mixing at 4˚C the mixture was washed by centrifugation 5 times using 1.5 ml buffer F plus 20 mM imidazole. All proteins, including his-tagged protein and any interacting proteins, were then eluted from Ni-NTA agarose beads with Buffer F (300 µl) containing 250 mM imidazole. To measure binding of FxsA, gp10A and gp1.2 to the immobilized PifA the resin was eluted stepwise with buffer F containing 50, 100,

150 mM NaCl. Each step eluate was subjected to SDS-PAGE, proteins specifically bound to his-tagged protein were analyzed by immunoblotting.

2). Column affinity chromatography:

a). Ni-NTA agarose: 1mg of His-PifA or SP6 his-RNA polymerase (RNAP) was gently mixed at 4˚C overnight with 0.2 ml of a 50% Ni-NTA agarose beads slurry in buffer F. After washing three times with 1.5 ml of buffer F, the resin was equilibrated with 20 mM Tris-HCl, pH 7.5, 10% glycerol, 0.1 mM EDTA, 0.1 mM DTT (buffer G) containing 25 mM NaCl and 0.5 mg/ml BSA. Gp10A (0.14 mg) or gp1.2 (0.5 mg) was mixed with 0.1 ml (drained volume) of PifA or RNAP resin in a total volume of 0.5 ml buffer G containing 25 mM NaCl and 0.5 mg/ml BSA. After incubation for two hours at 4˚C, the slurry was applied to a Pasteur pipette tip column. After washing with buffer G containing 25 mM NaCl and 0.5 mg/ml BSA to remove unbound gp10A or gp1.2, a step gradient of buffer G containing 50,100, 150, 200,250, 300, 350 or 400 mM NaCl (1 ml for each concentration) was used, at a flow rate of 1 ml per hr, to elute gp10A or gp1.2. Elution was performed at room temperature, and fractions of 200 µl were collected. The presence of gp1.2 and gp10A was monitored using the Bradford reagent and confirmed by SDS-PAGE.

b). Affi-Gel 10: His-PifA and SP6 his-RNAP were coupled to Affi-Gel 10 as recommended by Bio Rad. Each protein (0.5 mg in 0.25 ml) was dialyzed against 0.1M MOPS pH 7.5, 0.5 mM EDTA, 10% glycerol, and incubated at 4˚C

overnight with 0.3 ml of drained Affi-Gel 10 equilibrated in the same buffer. The resin was then saturated with ethanolamine and washed; coupling efficiency was 80% for his-PifA and 85% for SP6 his-RNA polymerase. Each resin (150 µl) was then gently mixed with his-tagged mutant gp1.2 (0.5 mg in 0.25 ml) in a total volume of 0.4 ml of buffer G containing 0.5% BSA at 4˚C for two hr. The resin was then loaded onto a Pasteur pipette tip column; after washing with buffer G containing 25 mM NaCl and 0.5 mg/ml BSA, bound mutant gp1.2 was eluted, collected and detected in the same manner as described above for wild-type gp1.2.

2. Co-immunoprecipitation

1). gp10A and gp1.2 interactions:

Purified gp1.2 (10 µg) was incubated with gp10A (10 µg) or his-PifA (10 µg) in 0.5 ml of 25 mM Tris-HCl pH 8.0, 25 mM NaCl, 0.1% NP-40 at 4°C for 6 hr with gentle mixing. Five µl of anti-gp10A or anti-PifA serum was added. After 1 hr 10 µl of a 50% protein agarose bead slurry was then added and incubation continued at 4°C for 1 hr. The agarose beads were finally collected and washed with the above buffer. SDS-loading buffer was added to the beads and the mixture was boiled for 5 min. Soluble materials were subjected to SDS-PAGE and the presence of gp1.2 was examined by immunoblotting using anti-gp1.2 antibody.

2) PifA-del(1-462) and truncated FxsA interactions:

BL21DE3(pLysS)(pHisFxsA111) was grown in M9 minimal medium containing 0.2% glucose, 1mM $MgSO_4$, 20 µg/ml vitamin B_1 at 37˚C to A_{600} =

0.25 and induced with 1mM IPTG for 30 min. Cells were labeled with 10 µCi/ml of [^{35}S]methionine (1175Ci/mmol) for 5 min and resuspended in 50mM K_2HPO_4, pH8.0, 1mM EDTA, 0.1 M NaCl, 0.1% Triton X-100 (100 µl per ml culture) and then lysed by freezing and thawing twice. After removal of cell debris by centrifugation, 0.1 ml of the supernatant was incubated with 1 ml of cell extract containing his-PifA-del(1-462) at 4˚C for 2 hr with gentle mixing. SP6 his-RNAP was used as a control. Ten µl of anti-PifA serum was added, mixed for 1 hr at 4˚C, and 50 µl of 33% protein A agarose beads was then added. Incubation was continued for 1 hr at 4˚C. The agarose beads were collected, washed with 50mM K_2HPO_4, pH8.0, 1mM EDTA, 0.1 M NaCl and boiled in SDS-loading buffer for 5 min. Soluble material was subjected to SDS-PAGE and the presence of FxsA was revealed by phosphorimagery.

3. Binding constant (Kd) assay

Purified gp10A (50 nM) or gp1.2 (100 nM) was incubated with increasing concentrations of his-PifA in a volume of 100 µl at 4˚C for 2 hours in binding buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM imidazole and 1mg/ml BSA). Forty µl of Ni-NTA agarose beads were added to each reaction and the mixture incubated for 1 hr to retrieve his-PifA. The resin was then washed five times with 1.5 ml ice cold 25mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM imidazole and 0.1% Triton X-100. Bound proteins were eluted with a 25 mM Tris-HCl, pH 7.5, 250 mM imidazole, 300 mM NaCl solution and were subjected to

SDS-PAGE. Gp10A or gp1.2 protein was detected by immunoblotting. The amount of gp10A or gp1.2 that bound to His-PifA in each reaction was determined by measuring the density of each band using the program NIH image.

[^{35}S] labeling of FxsA-phoA fusions

1 ml of CC118 containing a *fxsA-phoA* fusion plasmid was grown in M9 minimal medium supplemented with 0.2% glucose, 100 µg/ml arginine and leucine, 20 µg/ml vitamin B1 and 1mM $MgSO_4$, at 37˚C to A_{600} = 0.3 and pulse-labeled with 100 µCi/ml of [^{35}S]methionine (1175 Ci/mmol) for 1 min. Immunoprecipitation was performed as described (Ito, 1981). The cell pellet from 1 ml culture was dissolved in 20 µl of 1% SDS, 50mM Tris-HCl (pH8.0) and 1 mM EDTA and boiled for 2 min. Six hundred and sixty µl of cold Triton buffer (2% Triton X-100, 50 mM Tris-HCl, pH8.0, 0.15M NaCl, 0.1 mM EDTA) was added. After removal of a non-specific precipitate by microcentrifugation for 10 min at 4˚C, the supernatant was mixed with 2 µl of anti-PhoA antibody at 4˚C for 1 hr. Forty µl of 33% protein A agarose beads were then added and the mixture was incubated at 4˚C for 1 hr. Beads were pelleted, washed twice with Triton buffer and once with 10mM Tris-HCl, pH8.0, and then were boiled for 2 min in SDS-PAGE loading buffer. Soluble material was analyzed by SDS-PAGE.

Alkaline phosphatase activity

Alkaline phosphatase activities were measured by a modified procedure of Brickman and Beckwith (1975). Log-phase cells (1.5 ml) were resuspended in 1

ml 1M Tris-HCl pH 8.0 and the A_{600} was measured. Cells were permeabilized by the addition of 10 µl $CHCl_3$ and 10 µl 10% SDS, and then 50 µl of 80 mg/ml p-nitrophenyl phosphate (Sigma 104 phosphatase substrate) was added. The reaction was incubated at room temperature for 10 min and was terminated by the addition of 100 µl 1M K_2HPO_4. After removal of cell debris by centrifugation, the A_{420} of the supernatant was measured. Activity is calculated as units = 1000 x A_{420} /(t x A_{600}), where t = time of incubation (min). All assays were carried out using the strain CC118 containing a test plasmid. Background values were obtained from the strain containing the vector plasmid pSWFII and were subtracted from the value obtained with each PhoA-fusion.

Proteinase K accessibility

Preparation of spheroplasts: One hundred ml of IJ1200(pWF185), IJ1198(pHisFxsA) or Top10(pHisPifA) were grown at 37˚C to A_{600}= 0.7 and harvested by centrifugation. Cells were suspended in 10 ml of ice-cold 0.5 M sucrose, 100 mM Tris-HCl, pH 8.0, 5 mM EDTA. After addition of lysozyme to 80 µg/ml, an equal volume of ice-cold H_2O was added and the mixture was incubated on ice for 10 min. The formation of spheroplasts was monitored by microscopy to ensure that less than 10% of intact cells remained. After adding $MgSO_4$ to 20 mM, spheroplasts were collected using a microcentrifuge at 10,000 rpm for 1 min and were then resuspended in 10 ml of ice-cold 0.25 mM sucrose, 50 mM Tris-HCl, pH 8.0 10 mM $MgSO_4$ (Cao *et al.*, 1994).

Preparation of inverted membrane vesicles (IMV): One hundred ml of IJ1200(pWF185), IJ1200(pHisfxsA) or Top10(pHisPifA) cells were grown and harvested as described for spheroplasts. The cell pellet was suspended in 10 ml of 50 mM Tris-HCl, pH 8.0, 0.1 mM NaCl, 20 mM EDTA. Cells were disrupted by a single passage through a French pressure cell at 16,000 p.s.i. Unbroken cells and cell debris were removed by centrifugation at 5,000 x g for 10 min at 4˚C. This procedure results in the conversion of at least 95% of cells into inverted membrane vesicles (Grundling *et al.*, 2000; Blasi *et al.*, 1999).

Proteinase K treatment: One ml of a suspension of spheroplasts or IMV was treated with 1mg/ml proteinase K at 37˚C for various times; digestion was stopped by the addition of 2 mM PMSF. One sample was treated with 2% Triton X-100 before proteinase K treatment. Spheroplasts were collected by pelleting at 10,000 rpm in a microcentrifuge for 1 min and IMV were collected by centrifugation at 100,000 x g for 2 hr. Pellets were dissolved in 2 x SDS-loading buffer and boiled for 5 min for SDS-PAGE analysis. The presence of FxsA or PifA was determined by immunoblotting using antibody against the C-terminus of FxsA or anti-PifA antibody.

Examination of the effect of mutation *fxsA pec109* on the cell growth

Approximately 10^3 exponentially growing cells of each of the following strains, IJ1199 Nal^R, IJ1199 *fxsA::cat*, IJ1198 *fxsA::kan* and IJ1198 $T5^R$, were mixed and incubated in either LB or M9 minimal glucose at 30˚C or 37˚C to a

density of 2 x 10^8 cells /ml. Cultures were harvested and viable cells of each strain were counted as colonies on LB plates after selection for antibiotic or phage T5 resistance. The purpose of using this mixed culture was to magnify any growth defects of these strains by growing them in competition. For measuring the total number of viable cells when cultures reach saturation, 10^3 cells of each strain were grown separately under the same conditions as above until the culture was saturated. The total number of viable cells in each culture was measured as colony forming units after plating on LB plates. Approximately 10^3 exponentially growing cells of each of the following strains, IJ1199 Nal^R, IJ1199 *fxsA::cat*, IJ1198 *fxsA::kan* and IJ1198 $T5^R$, were mixed and incubated in either LB or M9 minimal glucose at 30˚C or 37˚C to a density of 2 x 10^8 cells /ml. Cultures were harvested and viable cells of each strain were counted as colonies on LB plates after selection for antibiotic or phage T5 resistance. The purpose of using this mixed culture was to magnify any growth defects of these strains by growing them in competition. For measuring the total number of viable cells when cultures reach saturation, 10^3 cells of each strain were grown separately under the same conditions as above until the culture was saturated. The total number of viable cells in each culture was measured as colony forming units after plating on LB plates.

Chapter 3. Results and Discussion

ISOLATION OF *E. COLI* MUTANTS THAT SUPPRESS F EXCLUSION

Because co-expression of *pifA* and T7 gene *1.2* or gene *10* causes cell death (Schmitt and Molineux, 1991a), an essential cellular function must be affected by the combination of the phage and plasmid proteins. It was reasoned that mutants of *E. coli* that tolerate coexpression of gene *pifA* and gene *10* or *1.2* would either be altered in this target function or would express a separate activity that could protect the cell from the lethal interaction of the host's and parasite's gene product. By selecting for tolerance to coexpression of gene *10* and *pifA*, Wang *et al.* (1999a) isolated a single *E. coli* mutant. This mutant contains the *fxsAp109* mutation which allows T7 to grow in the presence of F. However, disruption of *fxsA* by the insertion of a Kn^r cassette does not abolish the toxicity of the combination of PifA and T7 gp1.2 or t7 gp10, suggesting that FxsA is not the cellular target of the parasites' proteins.

A similar approach was taken to select for more *E. coli* mutants that tolerate coexpression of *pifA* and gene *10* or *pifA* and gene *1.2* in the hope of identifying the targeted cellular components. Mutants lysogenic for λ1.2pifK and λ10pifK were isolated but no mutant was obtained that clearly supported 434-1.2pifK growth. Nine candidate mutants were considered to have passed a patch

Table 3. Titers of *imm*434 derivatives on λ1.2pifK lysogens and λ⁻ derivatives

IJ1198[b](λ1.2pifK) Lysogen	Non-lysogen	Efficiency of plating[a]	
		434-1.2pifK	434-10pifK
	IJ1198	2.6×10^{-5}	3.2×10^{-5}
1		[c]	1.7×10^{-5}
	1a	1.7×10^{-5}	1.3×10^{-5}
2		1.1×10^{-4}	0.65
3		1.5×10^{-4}	0.57
	3a	1.1×10^{-4}	0.8
4		1.1×10^{-3}	6.6×10^{-4}
	4a	1.0×10^{-5}	5.4×10^{-6}
5		1.6×10^{-4}	0.77
8		1.8×10^{-4}	0.43
9		3.2×10^{-4}	7.7×10^{-4}
	9a	3.0×10^{-5}	1.6×10^{-5}
11		3.8×10^{-4}	1.0×10^{-3}
	11a	2.5×10^{-5}	1.4×10^{-5}
12		2.1×10^{-4}	1.1×10^{-3}
	12a	3.3×10^{-5}	1.2×10^{-5}

[a] Relative to IJ1142. [b] Mutant derivative. [c] Not done.

test using 434-1.2pifK but all grew poorly. These *imm*λ lysogens were then tested quantitatively for growth of 434-1.2pifK; none was fully permissive, although one (#4) gave plaques at a frequency of 100-fold that of the parent strain IJ1198 (Table 3).

Unfortunately, and for unknown reasons, the λ1.2-pifK prophage could not be cured by brief thermal inactivation of the *c*I857 repressor from any of the mutants. Even more surprisingly, three strains could not even be transduced by phage P1 to *imm*λ-sensitivity. However, no cured strain was able to support significant growth of 434-1.2pifK and even the increased plating efficiency of 434-1.2pifK on lysogen #4 was lost following removal of the prophage.

The lack of full sensitivity of these strains to 434-1.2pifK suggests that any mutation(s) they contain confers tolerance only to a low level of gp1.2 and PifA. Perhaps the increased level of *1.2* and *pifA* expression from the derepressed or excised prophage λ1.2-pifK is lethal to these cells or there is a second mutation that makes these strains dependent on the prophage which cannot, therefore, be cured.

Unexpectedly, four λ1.2-pifK lysogens, only one of which (#3) could be made non-lysogenic, supported the growth of 434-10pifK (Table 3). Therefore, survivors of a challenge with *pifA* and T7 gene *1.2* are also tolerant to gene*10* and *pifA*. Non-lysogenic strain #3a was transduced with P1 grown on IJ1203 (*fxsAp109*::Kn). All 48 Kn^r transductants tested became resistant to 434-10pifK,

suggesting that strain 3a contained a *fxsA* mutation. The chromosomal *fxsA* region of the four λ1.2-pifK-tolerant strains that also supported growth of 434-10pifK was then amplified and sequenced. All contained the same G→T mutation in the -10 region of the *fxsA* promoter. This is also the same mutation (*fxsAp109*), present in IJ1199, which was however isolated for tolerance to λ1.2-pifK.

Twelve independent mutants were selected as survivors of a gene *10* and *pifA* challenge. All clearly supported the growth of 434-10pifK but all were shown to contain at least two chromosomal mutations. Removal of any one mutation by transduction either prevented growth or allowed only very poor growth of 434-10pifK, but also removed all easily scored phenotypes, making further analysis problematic. In addition, these mutants could not serve as donors for transduction of the 434-10pifK-sensitive phenotype to a naïve strain and they were therefore not studied further.

PROTEIN LOCALIZATION IN *E. COLI*

1. Localization of PifA

To determine where PifA is localized in *E. coli*, a crude cell extract was fractionated into cytoplasmic and membrane fractions. PifA protein was detected using anti-PifA serum and was found exclusively in the membrane fraction (Fig. 3A). Treatment of the membrane fraction with 0.5% Sarkosyl resulted in the complete solubilization of PifA (Fig. 3B). As Sarkosyl selectively solubilizes

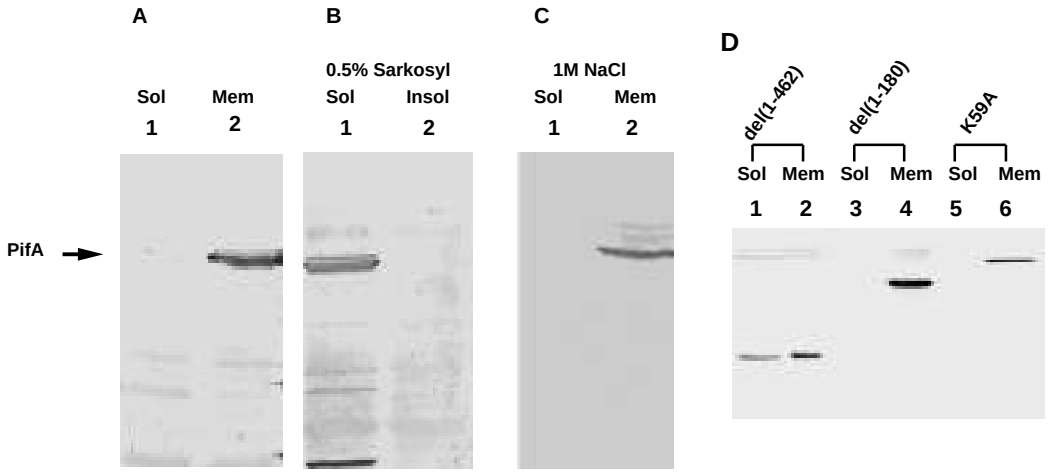


Figure 3. Localization of PifA(A, B, C) and N-terminal truncated or mutant PifA (D) in *E.coli*.

A crude cell extract of IJ512, Top10(pGX25), Top10(pGX26) or Top10(pGX31) was separated into membrane and soluble fractions, proteins were separated by SDS-PAGE, and PifA detected after immunoblotting using anti-PifA serum. A). Lane lane 1: soluble proteins; 2: membrane proteins. B). The membrane was extracted with 0.5% Sarkosyl. Lane 1: Sarkosyl-soluble; lane 2: Sarkosyl-insoluble. C). The membrane was treated with 1M NaCl. Lane 1: soluble proteins; lane 2: membrane proteins. D). Lanes 1, 2: PifA-del(1-462); lanes: 3, 4: PifA-del(1-180); lanes 5, 6: PifA K59A; lanes 1, 3, 5: soluble proteins; lanes 2, 4, 6: membrane proteins. Each lane contains 50µg of protein.

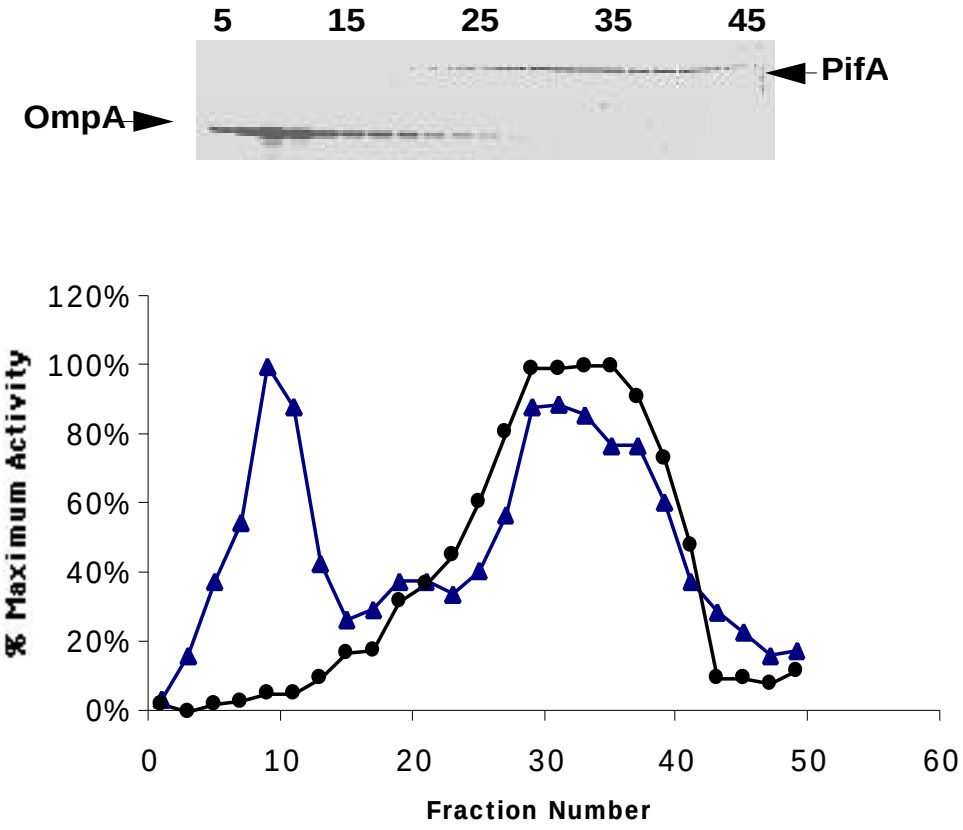


Figure 4. PifA fractionates as an inner membrane protein.

IJ512 inner and outer membranes were separated on a sucrose gradient. Fractions were assayed for protein content (▲) and NAH oxidase activity (●). Proteins in gradient fractions were also separated by SDS-PAGE (12%). PifA and outer membrane protein OmpA were determined by immunoblotting using antibodies to PifA and OmpA.

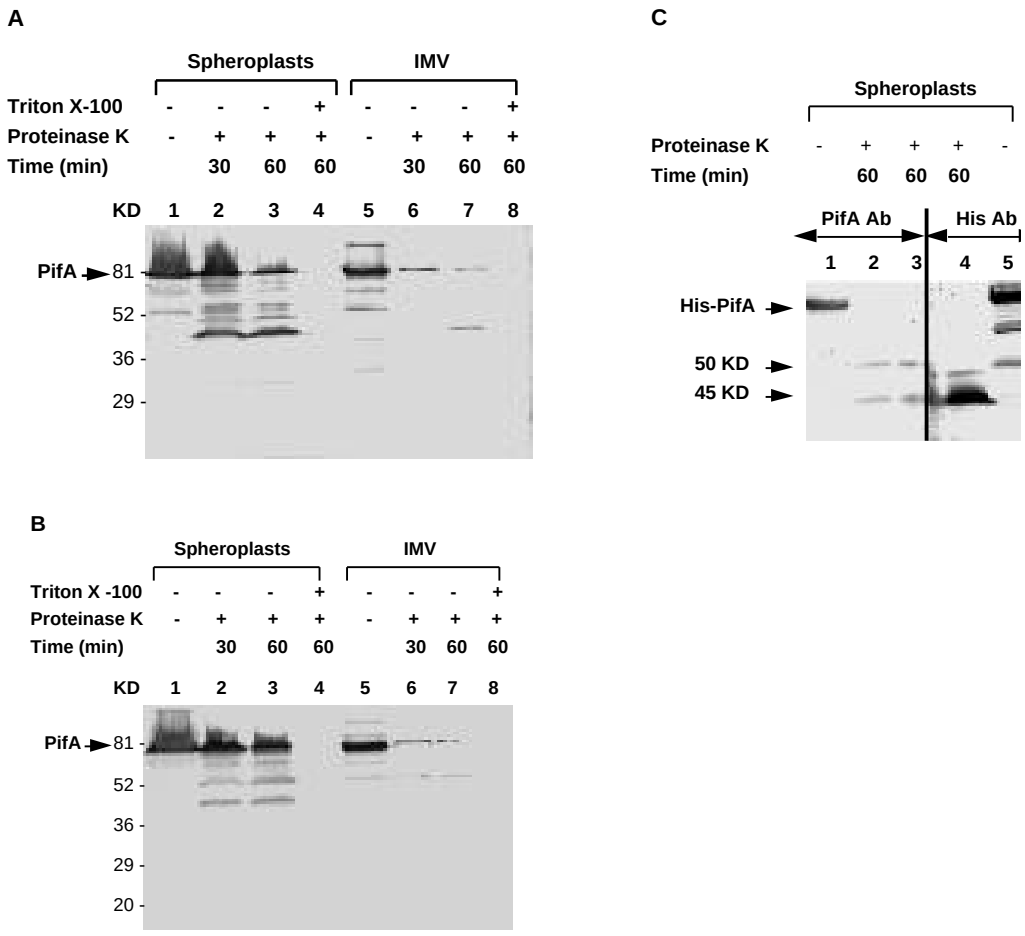


Figure 5. Protease accessibility of PifA in Spheroplasts and inverted membrane vesicles (IMV).

Spheroplasts and IMV prepared from Top10(pHispifA) were treated with or without detergent, with Proteinase K, for various times. Each sample was subjected to SDS-PAGE and analyzed by immunoblotting. A). His-PifA was detected with anti-histidine-tag antibody. B). His-PifA was detected with anti-PifA antibody. Lanes 1-4: spheroplast samples. Lanes 5-8: IMV samples. C). Spheroplast samples were transferred to one membrane, which was then cut into half from the middle of lane 3. One half of the membrane was incubated with anti-PifA antibody and the other half with anti-histidine-tag antibody.

cytoplasmic membrane proteins (Filip *et al*., 1973), this result strongly suggests that PifA is located in the cytoplasmic membrane. Washing the membrane with 1M NaCl did not remove PifA (Fig. 3C), indicating that it is not loosely associated via an ionic interaction. Localization of mutant proteins was also examined by immunoblotting of fractionated cell extracts. PifA-K59A and PifA-del(1-180) were found only in the membrane fraction (Fig. 3D); however, PifA-del(1-462) was detected in both the membrane (60%) and soluble fractions (40%). These results suggest that amino acid residues between codon 181 and 463 contain sequences associating with the membrane and some incomplete membrane localization sequences also likely exist distal to codon 462.

Membrane proteins were further separated into inner and outer membrane fractions through a 25-55% sucrose step gradient (Osborn *et al*., 1972). Two distinct peaks of protein were found (Fig. 4); as expected, OmpA, an outer membrane marker, was detected in the heavy fractions, whereas NADH oxidase, a cytoplasmic membrane protein, was found in the light fractions. The presence of PifA coincided with the inner membrane fractions, showing that PifA is associated with the inner membrane.

To determine whether PifA is an integral or membrane-associated protein, a protease accessibility assay was conducted. One major band (45kDa) was detected by anti-his-tag antibody in spheroplasts prepared from Top10(pHisPifA

(Fig. 5A). When the same spheroplast sample was immunoblotted by anti-PifA antibody, two major cleavage products of 45 and 50 kDa were observed although most PifA appeared protease-resistant (Fig. 5B). In contrast to spheroplasts, no major cleavage product of PifA was detected in inverted membrane vesicles (IMV), indicating that the major portion of PifA is in the cytoplasm. The fainter protease-resistance bands likely correspond to the small fraction of inner membrane vesicles that are not inverted.

A second experiment confirmed the presence of the 45 and 50 kDa fragments visualized by anti-PifA antibody but also showed that PifA was almost completely degraded by incubation with proteinase K (Fig. 5C). Anti-his-tag antibody also revealed a 45kDa fragment that migrated exactly with the fragment visualized by anti-PifA (Fig. 5C, lane 3). These results suggest that PifA might have a periplasmic domain(s) and the 45kDa band represents the N-terminal portion of PifA whereas the 50kDa band is C-terminal. However, the sum of 45 kDa and 50 kDa does not correspond to the molecular weight of PifA (80kDa). The fainter bands between 40 and 80 kDa (Fig. 5 A) and one between 50 and 80 kDa (Fig. 5 B) may result from either degradation or cross-reactions of the antibodies and other cellular proteins. It is not clear why the full length PifA (80kDa) was almost completely degraded in the second experiment (Fig. 5C, lanes 2 and 3) but not in the first (Fig. 5A and B). In addition, the combined intensity of the 45 and 50 kDa bands (Fig. 5C, lane 2) is much less than that of the untreated

PifA (80kDa) (Fig. 5C, lane 1). No strong conclusions on the membrane topology of PifA could be made from these experiments.

An alternative approach to determine whether PifA has transmembrane domain uses PhoA fusions. Various computer algorithms predict different, and some even predict no, transmembrane domains for PifA and there was no obvious region of the protein to target. A selection for Tn*phoA* insertions into a *pifA* plasmid was initially performed. More than 5000 Kn^R colonies were obtained, ten were blue but none contained an insertion in *pifA*. Eight contained an insertion in *bla*, the ampicillin resistance gene, one in *cat*, the chloramphenicol resistance gene (two different plasmid constructs were used in this study), and one in *pifC*. These results are surprising, a selection for antibiotic resistance had been maintained and thus both the *bla* and *cat* insertions resulted in active gene products. Furthermore, chloramphenicol acetyl transferase and PifC are both known to be cytoplasmic proteins.

Four *pifA-phoA* fusions were constructed using convenient restriction sites corresponding to PifA codons 121, 175, 275 and 520; an additional eight were made using oligonucleotides to provide fusions to the putative proteinase K cleavage region of PifA (codons 332 - 381). No fusion yielded blue colonies on indicator plates. The failure to find blue colonies suggests either that fusions to a periplasmic domain(s) of PifA are lethal, PifA has a very short periplasmic loop that was not disrupted by a Tn*phoA* insertion, or that PifA does not have a

periplasmic domain. This last idea contradicts the results from the protease accessibility assay. The necessity for high *pifA* expression for detection of PifA in fractionated membranes by immunoblotting, and the use of multicopy plasmids for the *phoA* fusion studies may have caused protein or fusion protein mis-localization. With higher titer antibodies it may be possible to conduct the protease accessibility assay using cells containing F as the source of *pifA*, and if a transmembrane topology is confirmed there would be justification for making new *phoA* fusions on single copy vectors. It is obvious that more data are needed to determine whether PifA is an integral or merely a membrane-associated protein.

2. Localization of T7 gp10A and gp1.2

Overexpression of *fxsA* suppresses F exclusion and it was shown that FxsA is an inner membrane protein (Wang *et al*., 1999a; 1999b). In that study, PifA was also shown to associate with the inner membrane. It was therefore of great interest to know where gp10 and gp1.2 are localized. To address this question, I carried out western blot analyses of fractionated cell extracts using anti-gp10A and anti-gp1.2 serum. Gp10 is largely insoluble in buffers containing low salt (Cerritelli and Studier, 1996a); to minimize aggregation or inclusion body formation of gp10A due to its low solubility, gp10A was expressed from a low copy number plasmid without induction. Gene *1.2* was expressed from a high copy number plasmid but was induced with a low concentration (0.1mM) of IPTG. Cell extracts were separated into cytoplasmic and membrane fractions. Both gp10A and gp1.2 were

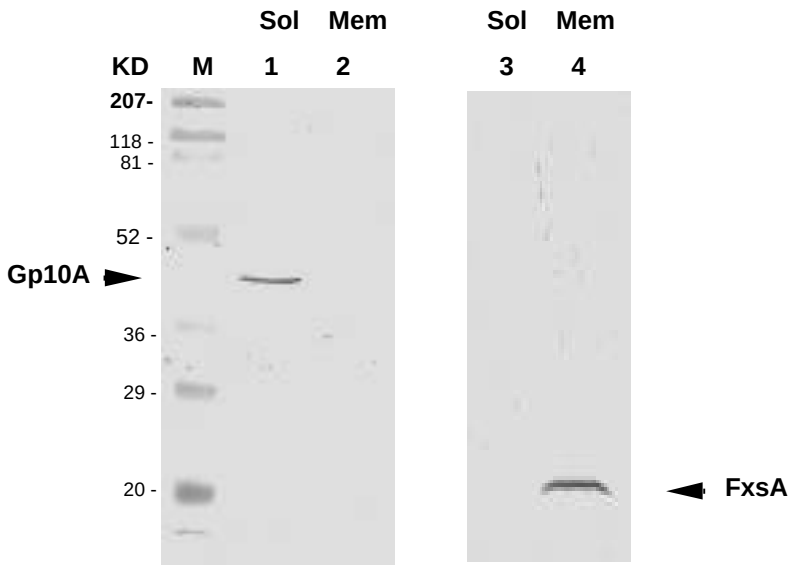


Figure 6. Localization of T7 gp10A.

A cell extract of IJ1199(pQ)(pWSK10A) was separated into membrane and soluble fractions, and proteins were resolved by SDS-PAGE. Gp10A and FxsA were detected by immunoblotting using anti-gp10A (lanes 1 and 2) and anti-FxsA serum (lanes 3 and 4). M: markers; lane 1, 3: soluble proteins; Lane 2, 4: membrane proteins. 25 μg of protein was loaded onto each lane.

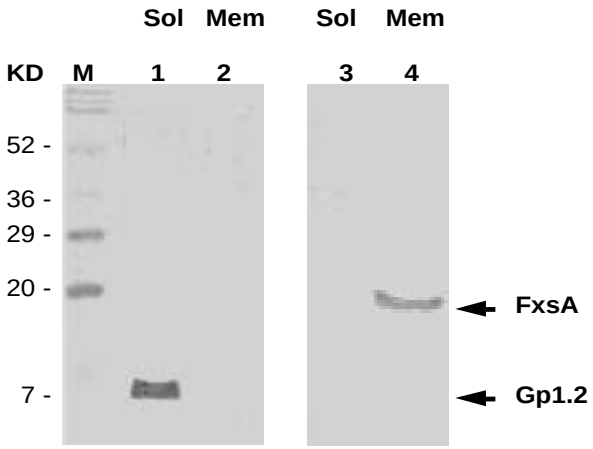


Figure 7. Localization of T7 gp1.2.

A cell extract of IJIJ1199(pQ)(pAK25) was separated into membrane and soluble fractions, proteins were separated by SDS-PAGE. Gp1.2 and FxsA were detected by immunoblotting using anti-gp1.2 (lanes 1, 2) and anti-FxsA serum (lanes 3, 4). M: markers; Lanes 1, 3: soluble proteins; Lanes 2, 4: membrane proteins. Each lane contains 25 μg of protein.

detected only in the cytoplasmic fraction (Figs. 6 and 7) indicating that both gp10A and gp1.2 are soluble proteins in *E. coli*. During membrane fractionation of gp10A- and gp1.2-containing cells, FxsA was only detected in the membrane fraction.

I also observed that 15-20% of gp10A fractionated with the membrane when gp10A was expressed from IJ1199(pWSK10A) after IPTG induction (data not shown). However, this small portion of gp10 was not detected with the membranes when NaCl (0.1M) was added to the cell extract before fractionation. Therefore, this apparent mis-localization of gp10A may be due to its insolubility or to artificially high levels of expression.

TOPOLOGICAL AND FUNCTIONAL STUDIES OF FXSA

1. Both N- and C-termini of FxsA cytoplasmic

Most computer algorithms predict that FxsA is an integral membrane protein with three transmembrane domains. An initial study of PhoA fusions suggested that FxsA has either three or four transmembrane domains, but the data were incomplete and some were hard to explain (Wang, 1998). To determine the proper membrane topology of FxsA, localization of its C-terminal domain was first investigated by protease accessibility assays in both inverted membrane vesicles and spheroplasts. In order to reduce the risk of mis-localization of FxsA due to

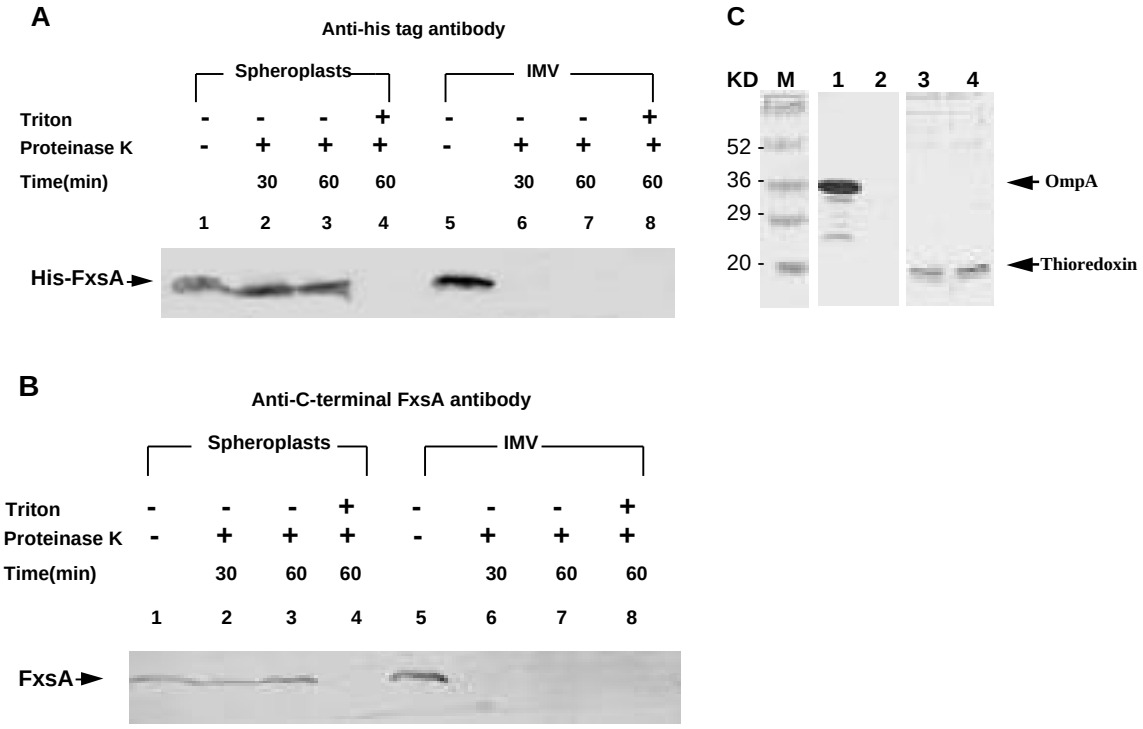


Figure 8. Both C- and N-termini of FxsA are cytoplasmic.

Spheroplasts and inverted membrane vesicle (IMV) were treated with Proteinase K, with or without detergent, for various times. Each sample was subjected to SDS-PAGE and analyzed by immunoblotting. A). N-terminal his-tagged FxsA was detected with anti-his antibody. B). FxsA was detected with anti-C-terminal FxsA antibody. Lanes 1-4: spheroplast. Lanes 5-8: IMV. C). OmpA is accessible to proteinase in spheroplasts. Lane 2: no proteinase K. Lane 3: proteinase K. Thioredoxin in spheroplasts was resistant to proteinase K. Lane 4: no proteinase K. Lane 5: proteinase K.

high level expression, FxsA was expressed from a low copy plasmid. The C-terminal domain of FxsA is susceptible to exogenous protease K in inverted membrane vesicles but not in spheroplasts (Fig. 8). Thus the C-terminus of FxsA is cytoplasmic. Complete digestion of OmpA by proteinase indicated a high efficiency of spheroplast formation, whereas the complete resistance of the cytoplasmic protein thioredoxin to external proteinase suggests that the inner membrane was not leaky.

A similar procedure was employed to determine the orientation of the N-terminus of FxsA. No antibody to the N-terminus of FxsA was made, in part because it was thought that this region comprises a transmembrane domain and would likely be insoluble. Therefore an N-terminal his-tagged FxsA was constructed; it is fully active *in vivo* as judged by the plating efficiency of T7 on IJ511/F´*lac* (Table 4). It is thus unlikely that the positively charged his-tag significantly alters the orientation of the native protein. Immunoblotting results using an anti-histidine tag antibody indicate that the his-tag at the N-terminus of FxsA is protected from protease K digestion in spheroplasts, but not in inverted membrane vesicles. Thus the N-terminus of FxsA is also cytoplasmic. As both termini of FxsA face the cytoplasm, and as previous work had established that FxsA spans the membrane there must be an even number of transmembrane segments. However, as no FxsA fragments could be detected after proteinase K digestion of spheroplasts any periplasmic segments must be short.

2. Alkaline phosphatase activities of FxsA-PhoA fusions

To investigate the membrane topology of FxsA, a series of *fxsA-phoA* gene fusions were constructed. Because alkaline phosphatase is only enzymatically active in the oxidizing environment of the periplasm and is inactive when cytoplasmic (Boyd *et al.*, 1989a), phosphatase activities of hybrid proteins correlate with the cellular location of the domain to which phosphatase is fused. However, simply finding phosphatase activities with a given fusion is not conclusive evidence that a particular domain is normally localized to the periplasm. Fusions to cytoplasmic domains of a protein nearly always show some activity due to a small amount of export (San Millan *et al.*, 1989). On other hand, low phosphatase activity does not necessarily mean that a domain is cytoplasmic because low levels of a hybrid protein could give the same result. Thus it is important to determine the efficiency with which various fusion proteins localize their phosphatase portion to the periplasm. In this study, specific activities were calculated by relating phosphatase activity to the amount of hybrid protein. Because hybrid proteins in which the phosphatase portion is localized to the cytoplasm are often unstable, specific activities were estimated by the rate of synthesis of the fusion protein in a pulse-label experiment. All fusion proteins were detected at their expected sizes and appeared to be synthesized at various rates (Fig. 9). Most fusions gave the expected full-length product although K53 showed low levels of degradation.

The fusions S18, A24, L84, P88 and L95 exhibit high levels of phosphatase (> 50 units) (Fig. 10). All fusions lie near predicted periplasmic domains of the four transmembrane segment model. Fusions S40, S45, V47, K53, P68, S77 and P122 show much lower activity (0.71-12.1 units) and are near predicted cytoplasmic domains. Two fusions (T32 and P102) display intermediate activities (18.3 and 29.2 units) and lie near the middle of predicted transmembrane segments. Therefore the model that FxsA has four transmembrane domains is strongly favored. The result is also in agreement with that of the protease sensitivity assay. The intermediate activities of T32 and P102 are probably because each is missing at least half the transmembrane segment and downstream cytoplasmic sequences. Without positively charged residues in cytoplasmic domains to serve as an anchor, phosphatase can be exported to the periplasm (Boyd *et al.*, 1987; San Millan *et al*., 1989).

In her initial characterization of FxsA topology, Wang (personal communication) made three independent fusions to the residue V47. On X-P plates, one gave dark blue, one gave pale blue and one gave white colonies. The results of both pulse-labeling and immunoblotting experiments showed that the fusion proteins from blue colonies were synthesized at 2.5 to 4.5 times higher rates than the fusion from the white colony but all specific activities were comparable. Thus, blue colonies and the resulting confusion about FxsA topology was due to increased synthesis of fusion proteins in some constructs. Notably, five fusions to

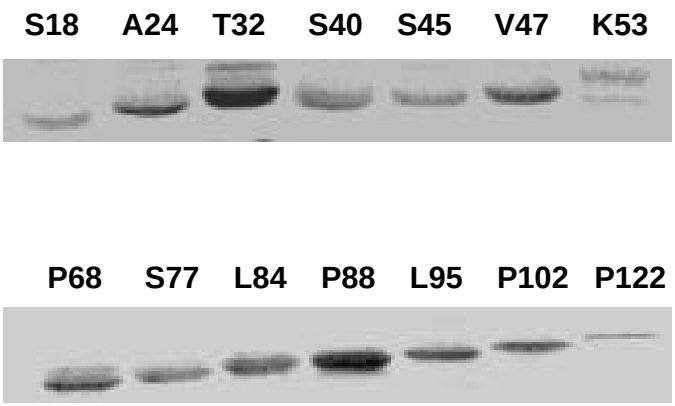
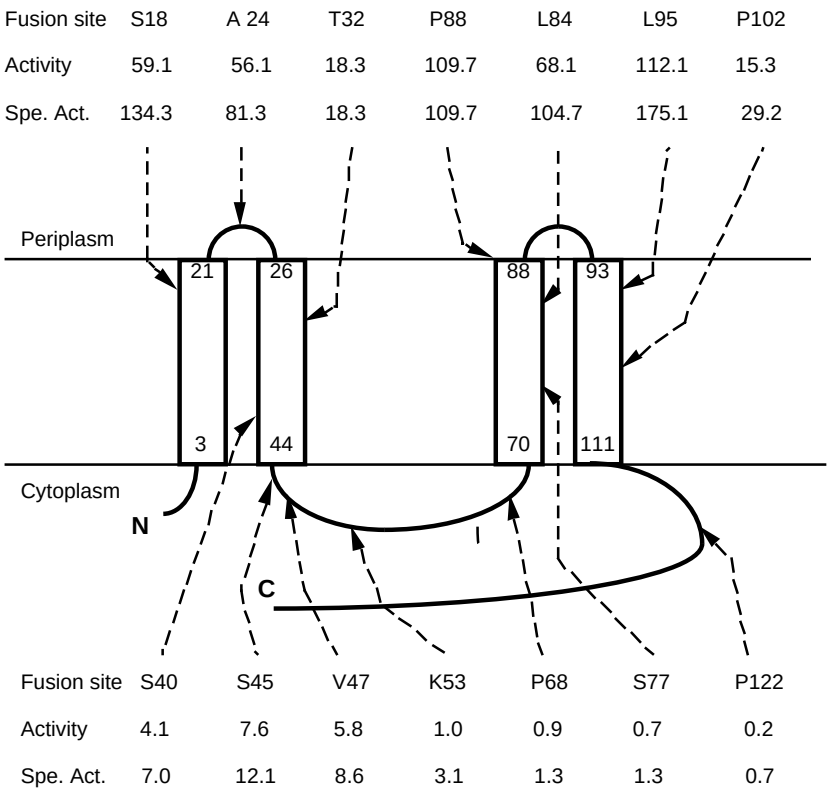


Figure 9. Immunoprecipitated pulse-labeled FxsAphoA fusion proteins.

FxsA-phoA hybrid proteins were precipitated with anti-alkaline phosphatase antibody after a 1-min pulse-label with [^{35}S] methionine and resolved on a SDS-PAGE gel and visualized by phosphorimagery.



B

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  1    MRWLPFIAIF  LYVYIEISIF  IQVAHVLGVL  LTLVLVIFTS  VIGMSLVRNQ
 50    GFKNFVLMQQ  KMAAGENPAA  EMIKSVSLII  AGLLLLLPGF  FTDFLGLLLL
100    LPPVQKHLTV  KLMPHLRFSR  MPGGGFSAGT  GGGNTFDGEY  QRKDDERDRL
151    DHKDDRQD
```

Figure 10. Alkaline phosphatase activities of *fxsA-phoA* fusions and a proposed membrane topology of FxsA.

A). Alkaline phosphatase activity of each *fxsA-phoA* fusion is shown with an arrow pointing to the site at which the fusion joint is located. All assays used the strain CC118 containing a *fxsA-phoA* plasmid. The specific activities were determined by dividing alkaline phosphatase activity by intensity of each band. The intensity of the T32 band was arbitrarily set at 1, and other bands were normalized to that value. B). Predicted FxsA sequence.

the same cytoplasmic loop of FxsA show significant differences in activities. The activities of fusions S40, S45, V47 are several-fold higher than those of K53 and P68. A possible explanation is that three positive charged residues (R48, K53 and K61) in the middle of the cytoplasmic loop may be crucial to anchor the second transmembrane stretch in the right orientation. Without these residues, a portion of phosphatase could be exported to the periplasm and provide activity. As the rate of synthesis of certain fusion proteins (e.g. V47) increases, more phosphatase is localized in the periplasm, resulting in an increased activity. A similar observation and explanation was reported for Mal F-phoA fusions (Boyd *et al*., 1987). Fusions of phosphatase to the beginning of a cytoplasmic domain of MalF (i.e., no cytoplasmic residues were included in the fusion) showed 7- to 20-fold higher activity than fusions to the end of the same cytoplasmic domain.

3. Membrane domains of FxsA are essential for alleviating F exclusion

Most computer algorithms predict that FxsA has three transmembrane domains. By using 19 residues as the length of a transmembrane segment some algorithms will predict four transmembrane domains and provide a structure that is in a good agreement with the results of the protease sensitivity assay and with the *phoA* fusion study. According to this model structure, the fourth transmembrane domain spans from glycine 96 to proline 114. To investigate the importance of the membrane domains of FxsA in protecting T7 from F exclusion and to determine the precise position of the last transmembrane domain, FxsA truncation mutants

Table 4. Relative plating efficiencies of T7 on strains containing plasmids synthesizing N-terminal fragments of FxsA.

Plasmids	Number of amino acids remaining in N-terminal FxsA	Relative plating efficiency[a] IJ511/F'*lac* / IJ511	
		30° C	37° C
pHisFxsA121	121	0.22	0.21
pHisFxsA115	115	0.4	0.66
pHisFxsA111	111	0.2	0.28
pHisFxsA107	107	0.04[b]	0.27[b]
pHisFxsA102	102	$< 10^{-7}$	$< 10^{-7}$
pHisFxsA	158(wild type)	0.37	0.26
pTrchisA	N/A[c]	$< 10^{-7}$	$< 10^{-7}$

[a.] Cells were grown to stationary phase in LB without IPTG induction.
[b.] Pinpoint plaques.
[c.] Vector plasmid.

lacking various parts of the normal C-terminus were created. The ability of the mutant proteins to suppress F exclusion was then examined. A FxsA truncation containing the N-terminal 111 of the normal 158 residues still allows T7 to plate normally in male cells (Table 4). FxsA containing only 107 residues also permits T7 to grow in male cells with essentially the same plating efficiency at 37˚C, although at 30˚C the plating efficiency decreases about 7-fold. Plaques on the FxsA107 host are also very small indicating that the mutant protein has partially lost activity. The FxsA truncation containing 107 residues is missing about one-third of the predicted fourth transmembrane domain, which is likely the cause for the partial loss of function. A deletion of 5 additional amino acids, providing a 102 residue FxsA, fails to support T7 to grow in male cells. The stability of each truncated FxsA (FxsA102, 107 and 111) was examined by immunoblotting using anti-his-tag antibody and were found to be stable (data not shown), indicating that the defect in their function is not due to an inability to accumulate in the membrane. Because a truncated FxsA containing 111 amino acids is fully functional, the last transmembrane domain likely terminates between histidine 107 and lysine 111. The latter may be important in anchoring the mutant protein in the membrane.

The results of this deletion study fit a four transmembrane segment model of FxsA better than a three segment structure. More importantly these data strongly

suggest that the membrane is the site where F exclusion of T7 occurs. Truncations removing the entire cytoplasmic C-terminal tail of FxsA remain fully active in alleviating F exclusion of T7. It can therefore be concluded that either the cytoplasmic loop between serine 45 and alanine 69 or the cytoplasmic membrane itself is the site where F exclusion of T7 development occurs. It would be interesting to mutate residues within the cytoplasmic domain and to determine the effects on the protective activity of FxsA. Alternatively, or perhaps in addition, transmembrane segment swaps with other known membrane proteins might prove highly informative.

4. The effect of *fxsAp109* mutation on cell growth

A promoter-up mutation in *E. coli fxsA* causes a 25-fold increase in expression of *fxsA* RNA (Wang *et al*., 1999a). It has been shown that *fxsA* is not essential for cell viability because *fxsA* insertions do not inhibit cell growth, but it is not known whether the presence or absence of *fxsA* affects the growth rate. Strains carrying various *fxsA* alleles were therefore grown in competition in the same culture under different conditions (Table 5).

Table 5. The effect of *fxsA* allele on cell generation time.

	Relevant genotype[a]	Generation time (min)			
		30° C		37° C	
		M9	LB	M9	LB
IJ1821	*fxsApec109 fxsA*$^{+}$ *Nal*^{R}	82.2	43.6	70.4	33.5
IJ1820	*fxsAp*$^{+}$ *fxsA*$^{+}$ *T5*^{R}	74.6	37.3	58.1	24.5
IJ1024	*fxsAp*$^{+}$ *fxsA::kan*	73.4	38.7	58.5	23.7
IJ1291	*fxsAp109 fxsA::cat*	74.4	36.7	57.9	23.4

a. All strains are derivatives of IJ1198.

Overexpression of *fxsA* in the strain IJ1821 causes a slower rate of growth in both rich and minimal medium, and at both 30˚C and at 37˚C. The generation times of IJ1821 are 6 and 7.6 min, respectively, longer than those of IJ1820 in LB and M9 minimal medium at 30˚C. At 37˚C, these differences increase to 8.9 and 12.3 min. IJ1199 grows at 43˚C; growth rate measurements were not performed at this temperature but it is expected that the differences in growth rate would be even greater. In contrast, a *fxsA* null mutation in either the IJ1198 or IJ1199 background does not manifest an overt detrimental effect on cell growth.

The number of viable cells of IJ1821 in saturated cultures was also compared with the isogenic *fxsA*$^{+}$ and *fxsA::Kn* strains IJ1820 and IJ1204. No significant difference was observed. Therefore, the *fxsAp109* mutation in IJ1199 retards the rate of cell growth, but allows cells to reach normal cell densities at saturation.

BIOCHEMICAL STUDIES

1. Protein purification

His-tagged proteins: As judged by staining with Coomassie Brilliant Blue, all his-tagged proteins were purified to near homogeneity by metal-chelate affinity chromatography (Fig. 11). The tagged proteins are all active in *vivo* as judged by phage plating or transformation assays (Tables 6, 7 and 8). Both his-gp1.2 and his-PifA are soluble in the buffer used. His-10A was soluble only at low

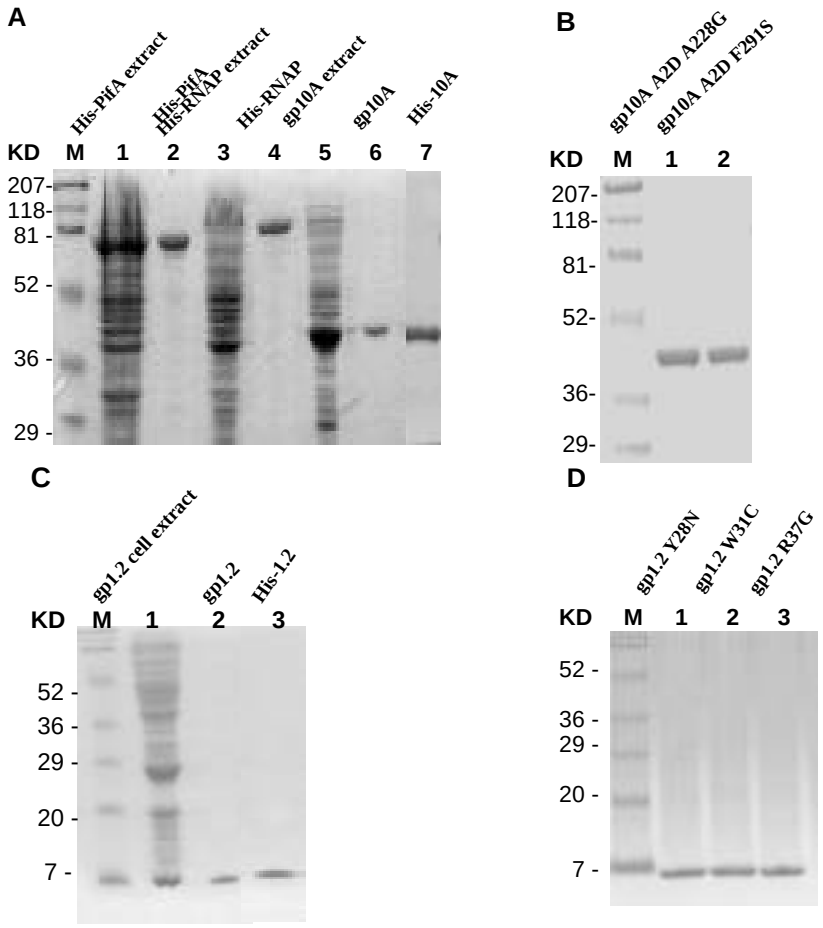


Figure 11. Protein purification.

Purified proteins were separated on A) and B) 12% (PifA, gp10A and FxsA) or C) and D)15% SDS-PAGE gels (gp1.2) and stained with Coomassie Brilliant Blue.

Table 6. Plasmids expressing 1.2 or 10A fusion proteins are toxic to strains containing F

Plasmid	genotype of T7 DNA [a]	Transformation efficiency [b] (F'*lac*/ F^-)
pAK25	*1.2*$^+$	0.0019
pGX107[c]	*his-1.2*$^+$	0.001
pGST1.2	*gst-1.2*$^+$	0.016
pAR1338	*10A*$^+$	2.5×10^{-4}
pHis-10A	*his-10A*$^+$	1.05×10^{-4}
pTrchisA		1
pKE11h		1
pGEX-3X		1
pUC 18		1
pBR322		1

[a.] Gene *1.2* is expressed from the T7Φ *10* promoter in pGX107, *tac* in pGST1.2 and *lac* in pAK25. Gene *10* is transcribed from *trc* in pHis-10A, T7Φ *10* promoter in pAR1338. Transformations were done under conditions in which the promoters were induced except for T7Φ *10* promoter in pAR1338.

[b.] Transformation efficiencies are calculated as the ratio of the number of transformant colonies obtained on IJ511/F'*lac* to those obtained on IJ511/F^-, normalized by the relative transformation efficiencies of the parent vector plasmids in each strain.

[c.] T7 RNA polymerase was supplied from the compatible plasmid pPK41. IJ511/F'*lac*(pPK41) and IJ511(pPK41) were used as male and female strains respectively.

Table 7. Complementation by plasmids expressing his-tagged mutant 1.2 proteins

Plasmids	Genotype of T7 DNA	EOP of T7 on IJ511(pPK41)/F'*lac* Containing a his-tagged mutant gene *1.2* plasmid [a]
pGX105	*1.2 Y28N*	0.6
pGX106	*1.2 W31C*	1.03
pGX109	*1.2 R37G*	0.86
pKE11H[b]		$<10^{-7}$

[a] EOP (Efficiency of Plating) is relative to the female strain IJ511(pPK41). pPK41 supplies T7 RNA polymerase gene.
[b.] Parent vector plasmid.

Table 8. Plating efficiency of T7 on IJ511(pHisPifA)

Plasmids	Genotype	Plating efficiency [a]
pHisPifA	***pifA***$^{+}$	$<10^{-6}$

[a] Relative to IJ511(pTrchisA), pTrchisA is parent plasmid vector. Expression of *pifA* is under *trc* promoter control, cells were grown without induction.

Table 9. Concentrations of gp1.2, gp10A and PifA in *E. coli*

	IJ511	IJ511/F'*lac*
	μM	μM
gp1.2	1.5	0.7
gp10A	49.5	2.1
PifA		8.3

concentrations.

T7 gp10A: The T7 capsid protein 10A is largely insoluble when concentrated cell extracts are made, but is mostly soluble if cells are lysed at a concentration of 10^{10} cells/ml in 20 mM Tris-HCl, pH 8.0, 3 mM β-mercaptoethanol, 10% glycerol, 2 mM EDTA, 100 mM NaCl, 0.1% Triton X-100 (Cerritelli and Studier, 1996a). Taking advantage of its low solubility, 20% ammonium sulfate could be used to precipitate gp10A, a concentration where most cellular proteins are still soluble. The solubilized ammonium sulfate pellet containing gp10A was then purified to homogeneity by DEAE ion exchange chromatography; gp10A flows through the resin to which most cellular proteins bind (Fig. 11A). Purified gp10A can only be kept soluble at ≥0.3 mg/ml in 20 mM Tris-HCl, pH 8.0, 3 mM β-mercaptoethanol, 10% glycerol, 2 mM EDTA, 100 mM NaCl. No reagent or condition has been found to increase significantly the solubility of gp10A.

T7 gp1.2: After induction with IPTG gp1.2 becomes a major fraction of the total cell protein. Purified gp1.2 was nearly homogeneous as judged by SDS-PAGE after staining with Coomassie Brilliant Blue (Fig. 11C and 11D).

2. Quantitation of gp1.2, gp10A and PifA in *E. coli*

PifA, gp1.2 and gp10 are the three proteins known to be responsible for F exclusion of T7. Previous data suggested that the concentrations of these proteins in each infected cell are directly related to the severity of exclusion. To gain more understanding of the mechanism of F exclusion, the amounts of gp1.2 and gp10A

in infected cells and the amount of PifA produced from the F plasmid were measured by quantitative immunoblotting. For quantification of gp1.2 and gp10A, cells were infected with T7 and harvested before cell lysis. IJ511/F'*lac* was used to measure PifA concentrations. Cell extracts were immunoblotted and quantified using the corresponding purified proteins as standards. Although rates of synthesis of T7 proteins are not known to be affected by the multiplicity of infection, in order to avoid possible complications of gene dosage resulting from varying numbers of phage genomes in cells, gp10 levels were measured in cells infected at an m.o.i. of 0.4. This multiplicity gives the maximum percentage (33%) of cells being infected and where almost every infected cell receives only one phage. Unfortunately, at this m.o.i. the quantity of gp1.2 produced in F^+ cells was too low to be measured accurately. Thus, gp1.2 in both F^- and F^+ cells was measured at an m.o.i. of 5.

T7 gp1.2, an early protein, is synthesized at relatively low levels as judged by protein accumulation in an F^- strain (Table 9). About 1500 copies of gp1.2 per cell corresponds to 1.5 μM. In F^+ cells, where exclusion of T7 inhibits late protein synthesis in particular, the concentration of gp1.2 is decreased about 50%. Because gene *1.2* is transcribed both early and late during infection and as gp1.2 itself causes exclusion, some inhibition of protein synthesis is not unexpected. The reduction in gp1.2 accumulation is comparable to that found in earlier studies. Morrison *et al.* (1974) observed in F^+ strains that the early protein gp1 was

synthesized at 75% the rate of F^- strains. Britton and Haselkorn (1975) and Whitaker *et al.* (1975) reported that gp1 and gp1.3 synthesis in F^+ strains was 40-60% and 30-40%, respectively, that of an F^- strain. However, T7 RNA polymerase (gp1) is only made early after infection and its synthesis would not be expected to be severely inhibited. Gp1.3 is made both early and late, like gp1.2, but these early studies measured its rate of protein synthesis by pulse-labeling with [^{35}S]methionine. Uptake of amino acids is affected during F exclusion of T7 (Schmitt *et al.*, 1991) and thus incorporation of radioactive amino acids cannot be used in a quantitative manner.

As expected for a major capsid protein, gp10A is extremely abundant in an F^- strain (Table 9). As each virion contains 415 copies of gp10, 49.5µM gp10A is sufficient to yield 150 progeny phages per cell. This value is within the range of the T7 burst (100–200) in permissive hosts, thereby confirming that the immunoblotting procedures employed are accurate. In contrast to gp1.2, the amount of gp10A synthesized in male cells is reduced by 95%. A concentration of 2.1 µM would allow a burst of 6 phage particles/cell, which is comparable to that found in a closely related F^+ strain by Spence *et al.*, (1983). Morrison *et al.* (1974) reported that no gp10 was detected in F^+ strains, but Condit and Steitz (1975) showed that the total amount of gp10A in F^- cells was 48-63% of F^+ cells. The result from this work is different from both earlier studies. The differences may be ascribed to the techniques employed here; Morrison *et al.* pulse-labeled proteins in

the interval of 13-15 min after infection and recovered by immunoprecipitation using anti-T7 antibody, whereas Condit and Steitz compared levels of pulse-labeled proteins from 6-10 min after infection. In this study the amount of gp10A was measured as the total accumulation during 20 min of infection. In addition, the F plasmid inhibits T7 to different levels when present in different host backgrounds and the use of different *E. coli* strains by the various researchers may also lead to differences in the degree of inhibition of late protein synthesis.

3. Protein interactions

1). PifA interacts with gp10A, gp1.2 and FxsA

a). Genetic studies showed that in the absence of phage infection, the combination of either gp10A or gp1.2 and PifA causes cell death (Schmitt and Molineux, 1991). It was therefore reasoned that PifA interacts both with gp10A and with gp1.2, and that each interaction initiates a chain of reactions leading to death of the phage-infected cell. Clearly a host function is attacked by the combination of PifA and either gp10 or gp1.2 during the abortive infection. However, this host target has not yet been identified. Overexpression of FxsA protects T7 from F exclusion but FxsA is known not to be the cellular component targeted by PifA and a T7 protein. Although the specific mechanism of this process remains unknown, several hypotheses can be advanced. T7 gp10 or gp1.2 may interact directly with PifA, the combination then interacts with the cellular

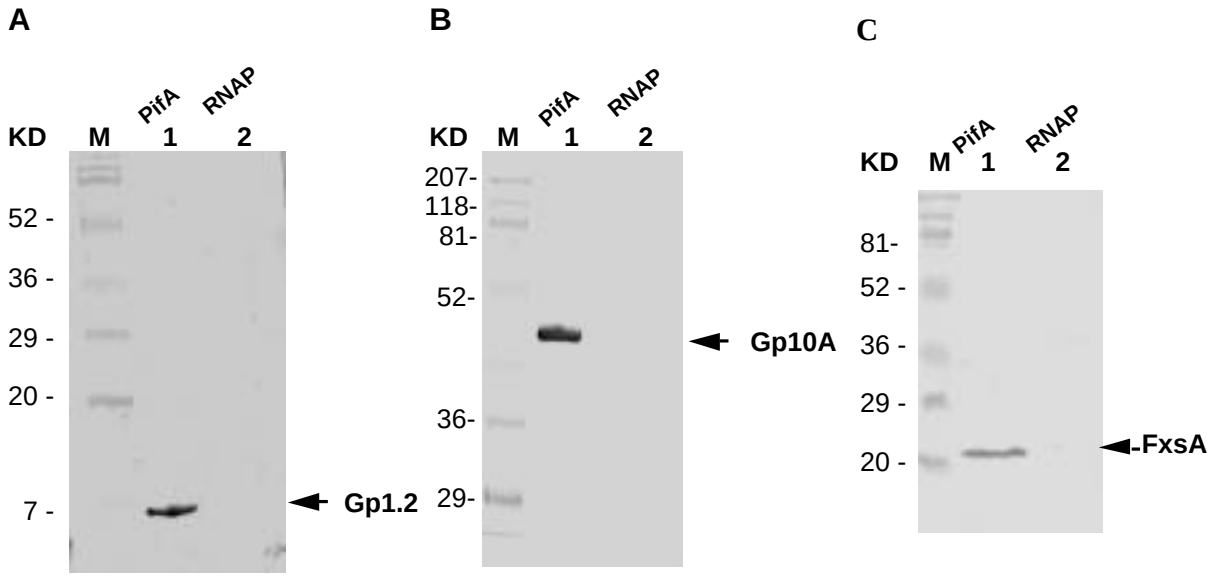


Figure 12. PifA interacts with T7 gp1.2, gp10A and FxsA.

His-PifA resin was incubated with gp1.2 (A), gp10A (B) and FxsA (C) cell extract. The eluatewas analyzed by immunoblotting using anti-gp1.2, anti-gp10A or anti-FxsA serum.

target; a T7 protein (gp10 or gp1.2) may interact with the cellular target, which then interacts with PifA; or vice versa, PifA may initially interact with the cellular target. In addition, gp10 or gp1.2 and PifA may each interact separately with the cellular target. The potential functions of FxsA in alleviating exclusion are equally variable. For example, FxsA may interact with PifA and/or the cellular target to prevent them from interacting with gp10 and gp1.2.

To investigate whether PifA physically interacts with gp10A, gp1.2 or FxsA and to try and identify the cellular component targeted by these proteins, a PifA affinity resin was prepared. Cell extracts containing gp10A, gp1.2 or FxsA were then incubated with the resin as described in Materials and Methods. After removal of unbound material, all specifically bound proteins were eluted. A comparable resin containing SP6 RNAP as the affinity ligand was used as a negative control. Gp10A, gp1.2 and FxsA were found to bind PifA (Fig. 12), indicating that all three interact specifically with PifA. To determine whether these interactions occur independently of any *E. coli* molecule, purified gp10A and gp1.2 were chromatographed on the PifA resin. Gp10A and gp1.2 bind to PifA in the absence of any *E. coli* component, suggesting that both gp10A and gp1.2 can bind directly to PifA. Other than FxsA no *E. coli* protein was found that bound PifA, and even this interaction could not be detected without overexpression of *fxsA*. (data not shown). This last observation implies that any interaction between

PifA and its cellular target is weak, that the target is not a soluble component of the cell, or that its concentration is below detection levels.

To determine the strength of the interaction with PifA and gp10A, and with PifA and gp1.2, a step gradient of NaCl was used to elute proteins from the PifA resin. Gp1.2 eluted at 250 mM NaCl (Fig. 13), whereas gp10A eluted between 300-350 mM NaCl (Fig. 14). Using a comparable resin bound with SP6 RNAP both gp1.2 and gp10A eluted with the washing buffer (25mM NaCl). Thus both gp1.2 and gp10A interact specifically with PifA, but gp10A binds with a higher affinity. The strength of the interaction between PifA and gp1.2 or gp10A correlates well with the toxicity of the phage proteins in male cells. Transformation efficiencies suggested that gp10A is more detrimental than gp1.2 to cells containing F (Schmitt *et al*., 1991). The present study confirms that observation and also shows that a complex of PifA and gp10A or of PifA and gp1.2 can be disrupted by high ionic strength, indicating that the protein-protein complex is maintained by ionic interactions.

The binding affinity of FxsA for PifA was also estimated by measuring the concentration of NaCl required to elute FxsA from PifA resin. Because no purified FxsA was obtained (due to the toxicity of *fxsA* overexpression) a cell extract was used to provide FxsA. The protein was eluted with 150mM NaCl (Table 10); the salt sensitivity of FxsA binding to PifA indicates that these proteins also interact through ionic bonds.

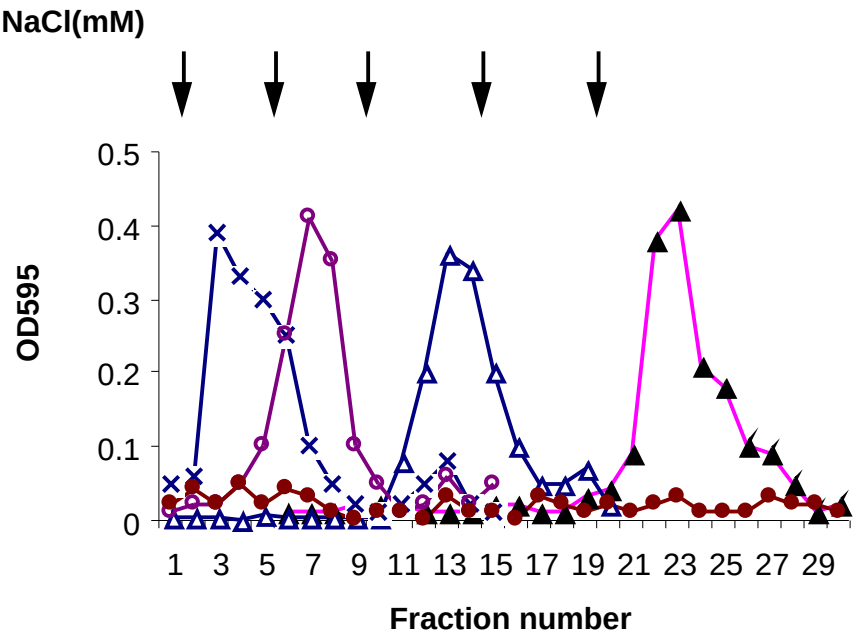


Figure 13. Elution of gp1.2 from a PifA resin.

Mutant and wild type gp1.2 were incubated with his-PifA bound to Affi-Gel 10 resin and eluted with a step gradient of NaCl. ×: gp1.2 Y28N; o: gp1.2 W31C; Δ: gp1.2 R37G; ▲: wild type gp1.2; ●: SP6 his-RNAP.

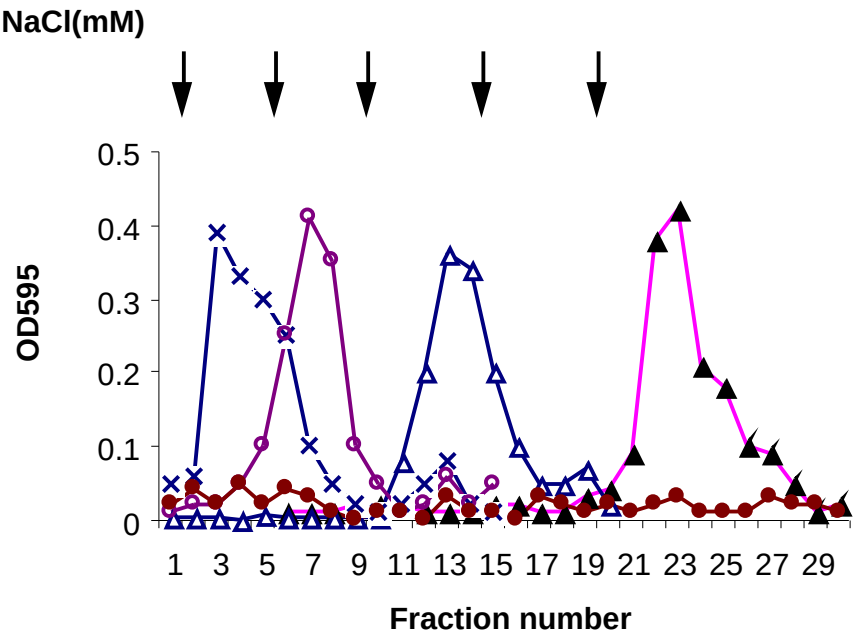


Figure 14. Elution of gp10A from a PifA resin.

Mutant and wild type gp10A were incubated with his-PifA bound to Ni-NTA agarose and eluted with a step gradient of NaCl. ●: gp10A A2D A228G; Δ**:** gp10A A2D F291S; ▲: wild type gp10A; ○: SP6 his-RNAP.

Table 10. The binding affinity of FxsA to his-PifA, his-10A and his-1.2

His tagged proteins	Concentration of NaCl required to elute FxsA[a]
His-PifA	150 mM
His-10A	100 mM
His-1.2	100 mM

[a] His tagged proteins were bound to the Ni-NTA affinity resin and incubated with cell extract containing FxsA. The resin was eluted with buffer containing NaCl. FxsA was detected by immunoblotting.

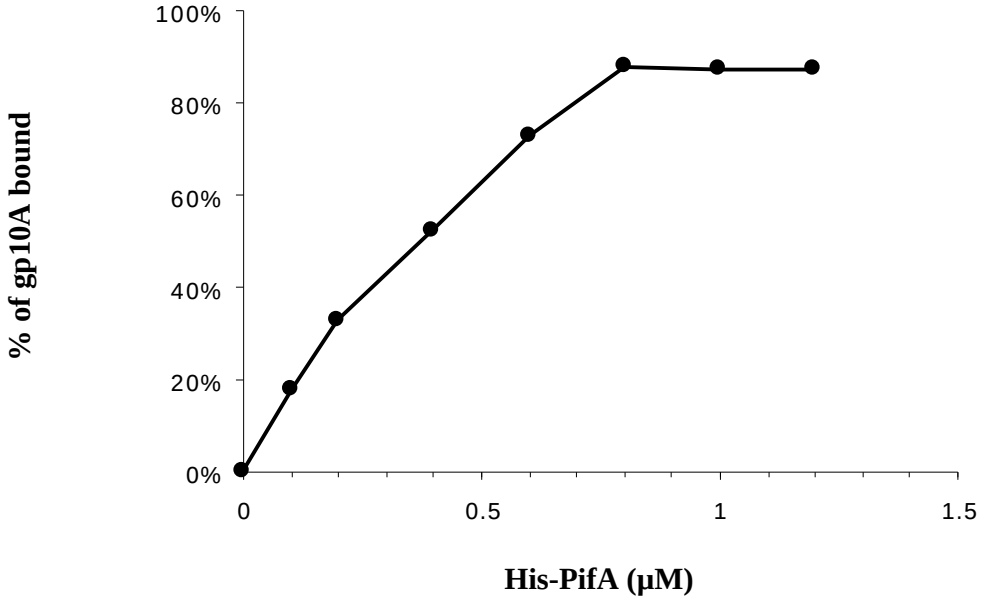


Figure 15. Titration of gp10A with his-PifA.

Various concentrations of his-PifA were incubated with 50nM gp10A. The amount of gp10A bound was determined by immunoblotting.

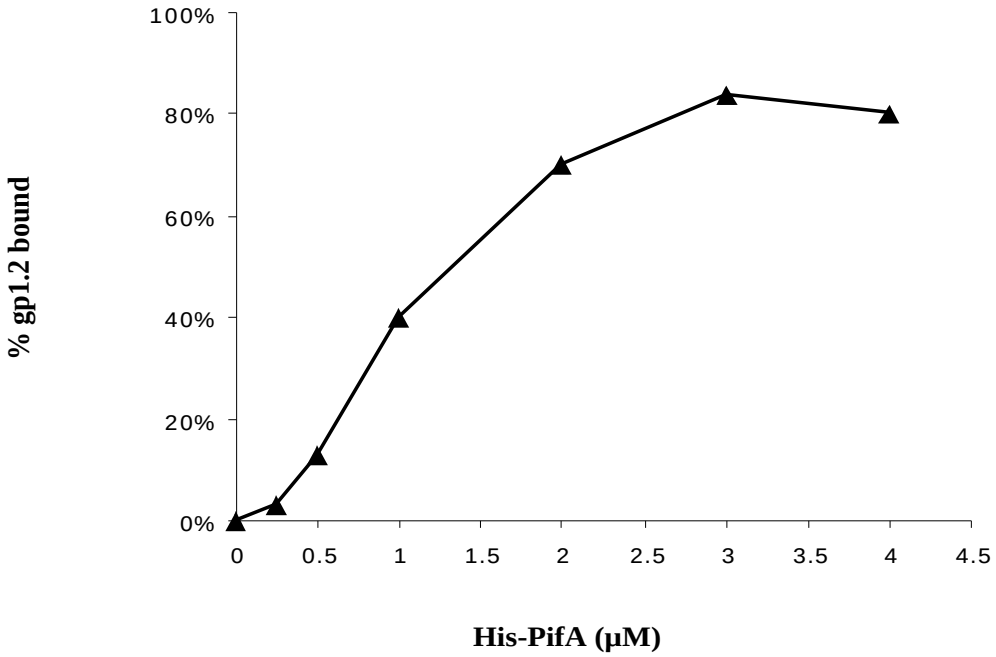


Figure 16. Titration of gp1.2 with his-Pif A

Varying concentrations of his-PifA were incubated with 100nM gp1.2. The amount of gp1.2 bound was determined by immunoblotting (see Materials and Methods for details).

The binding affinity of his-PifA to gp10A or gp1.2 was examined further by measuring equilibrium dissociation constants (Kd). To determine each Kd, gp10A or gp1.2 was pre-incubated with increasing concentrations of his-PifA as described in Materials and Methods. Complexes and unbound his-PifA were recovered using Ni-NTA agarose beads. The resin was then washed and proteins were eluted with 250 mM imidazole. Eluted proteins were electrophoresed on a SDS-PAGE gel, electrotransferred to a PVDF membrane and probed using anti-gp10A or anti-gp1.2 antibody. The titration curves were obtained after the quantification of each protein band (Figs. 15 and 16). Kd is determined as the concentration of his-PifA at which 50% of gp1.2 (or gp10A) bound. Apparent Kd values were 0.3 μM for his-PifA and gp10A and 1 μM for his-PifA and gp1.2. These values confirm the conclusion made earlier: that gp10A binds to PifA with a higher affinity than gp1.2. Complex formation would seem to be physiologically significant as the concentrations of PifA (8.3 μM) and gp10A (2.1 μM) in each female cell are greater than the Kd value for their interaction and the concentration of gp1.2 (0.7 μM) is only slightly less than the Kd value for its interaction with PifA.

b). Gene *10* mutations reduce the interaction with PifA. All T7 mutants that escape F exclusion contain at least two missense mutations in gene *10*. A T7 gene *1.2* deletion mutant carrying these gene *10* mutations plaques on a male strain at relatively normal efficiencies (Molineux *et al.*, 1989). Three suggestions were made to explain this observation. The mutations reduced the rate of capsid protein

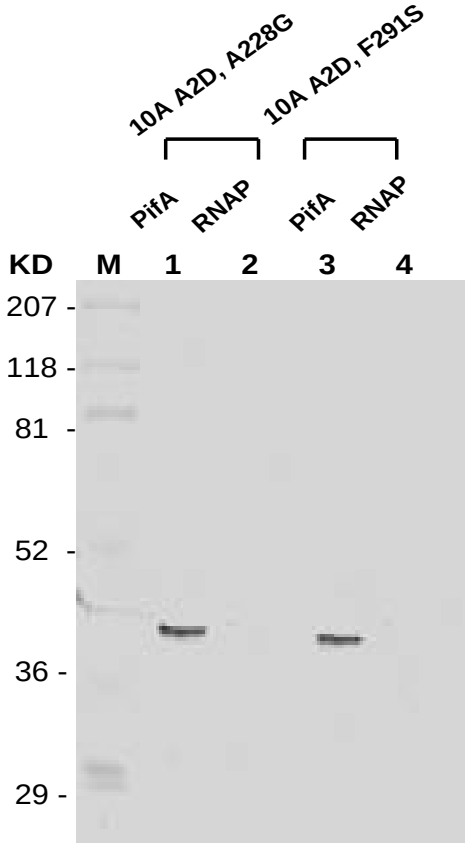


Figure 17. Mutant gp10A interacts with PifA.

Cell extracts containing mutant gp10A were incubated with his-PifA (lanes 1, 3) or SP6 his-RNAP (lanes 2, 4) and the eluates were immunoblotted.

synthesis, perhaps also making the time after infection at which the capsid protein reaches a critical concentration later, and the nature of interaction between capsid protein gp10 and PifA was altered.

To address these ideas more directly, the expression levels of two mutant capsid proteins, gp10A-A2D,A228G and gp10A-A2D,F291S were compared with that of the wild type at both 30˚C and 37˚C by immunoblotting (data not shown). Relative to wild-type gene *10* expression from the plasmid pET10A, mutant gene *10* expression from the equivalent plasmids pET10PC (*10A-A2D,A228G*) and pET10JT (*10A-A2D,F291S*) were reduced by 45% and 50% respectively at 30˚C. At 37˚C pET10PC and pET10JT expressed the mutant *10A* at only 65% and 53% the rate of wild-type.

Gold *et al*. (1981) reported that the presence of GCU (alanine) as the second codon correlates with highly efficient translation. The eight most actively synthesized T7 proteins have GCU as their second codon (Dunn and Studier, 1983). Thus the change of alanine to aspartic acid at codon 2 present in both gene 10 mutants is likely the cause of the reduced gene expression.

To examine whether the gene *10* mutations also affect the strength of the interaction of capsid protein with PifA, batch affinity chromatography was performed using immobilized his-PifA and extracts of cells containing gp10A-A2D,A228G or gp10A-A2D,F291S. Both mutant capsid proteins bound to PifA (Fig. 17). Binding strengths were assessed by measuring the concentration of NaCl

necessary for elution. Both mutant proteins eluted at 150-200mM NaCl (Fig. 14), a significantly lower concentration than required for wild type gp10A. This result suggests that both mutant capsid proteins interact with PifA with diminished affinity. Although a contribution from the codon 2 change cannot be excluded, the elution profiles of the two mutant proteins are different, suggesting that the reduced binding is due to the second gene *10* missense mutation.

The gene *10* mutations necessary for T7 to avoid F exclusion therefore not only lower the expression level of gp10 but also reduce binding to PifA, and the hypothesized reduced toxicity of the mutant gp10A is likely the result of both. However, plasmids containing these mutant gene *10* alleles remain as incompatible with F as wild type gene *10* in a transformation assay (Schmitt, 1991). Presumably this assay is more sensitive to toxic proteins than is phage growth.

c). Gene *1.2* mutations reduce binding of gp1.2 to PifA. In addition to gene *10* mutations, T7 must contain a missense or null mutation in gene *1.2* to escape F exclusion. It has been shown that plasmids containing the mutant gene *1.2* transform a male strain at essentially the same efficiency as the isogenic female strain (Schmitt and Molineux, 1991). It was concluded that the gene *1.2* mutation diminishes or abolishes the toxicity of gp1.2 towards *pifA*$^{+}$ cells. To show directly that reduced toxicity is due to a reduced affinity of the mutant gp1.2 for PifA, three mutant 1.2 proteins, gp1.2Y28N, gp1.2W31C and gp1.2R37G, were examined for binding to PifA. Cell extracts containing each mutant gp1.2 were incubated with

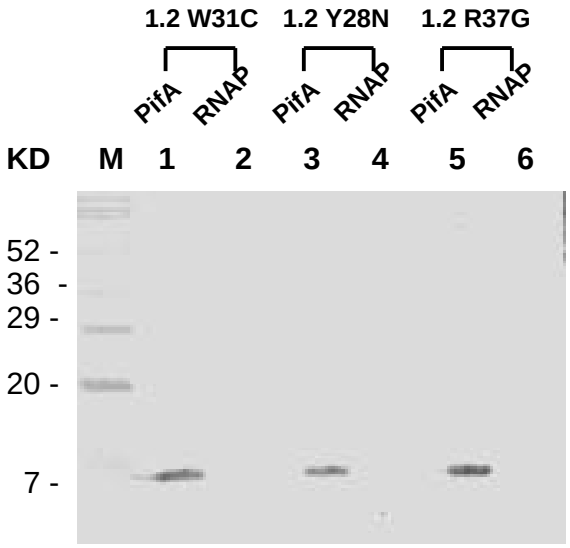


Figure 18. Mutant gp1.2 interactions with PifA.

Cell extracts containing mutant gp1.2 were incubated with his-PifA (lanes 1, 3, 5) or SP6 his-RNAP (lanes 2, 4, 6) resin. The eluates were subjected to SDS-PAGE and immunoblotted.

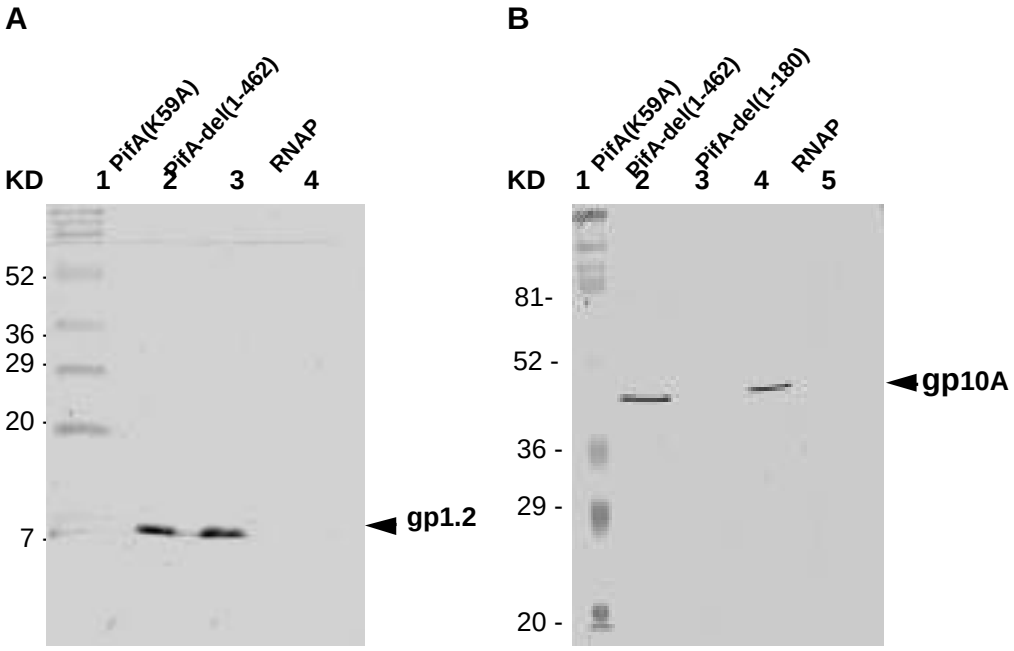


Figure 19. Interactions of mutant or N-terminal truncated PifA with gp1.2 (A) and gp10A(B).

His-PifA resin was incubated with purified gp1.2 or gp10A and the eluate was analyzed by immunoblotting using anti-gp1.2 or anti-gp10A serum.

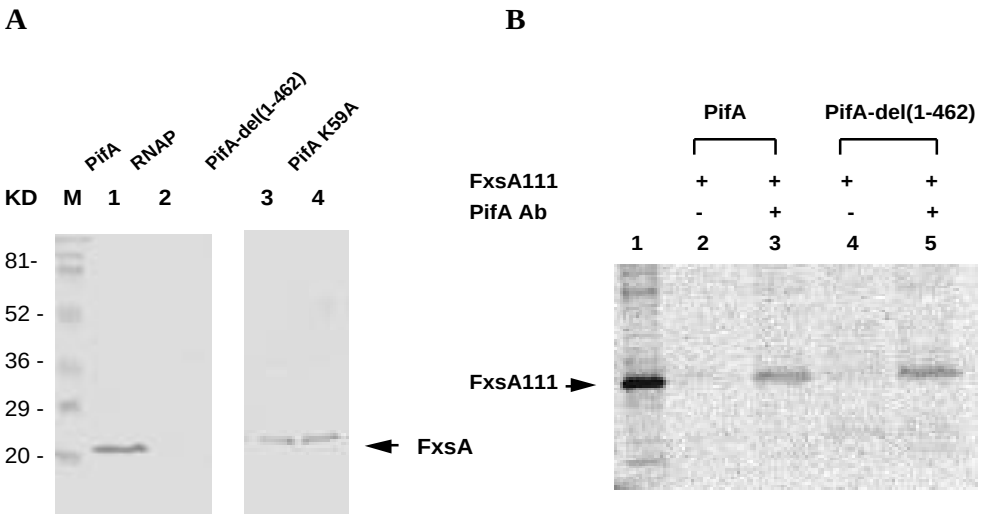


Figure 20. Interaction of PifA with FxsA.

A). PifA resins were incubated with FxsA-containing cell extracts. Eluate from each resin was subjected to SDS-PAGE and analyzed by immunoblotting. Lanes 1 and 2 are duplicate of Fig 10C.
B). PifA or PifA-del(1-462) was incubated with [^{35}S] labeled his-FxsA truncation containing its N-terminal 111 residues (FxsA111) and precipitated with anti-PifA antibody. Lane 1: immunoprecipitaed FxsA111 with anti-his-tag antibody.

immobilized his-PifA. After washing, bound proteins were eluted and subjected to immunoblotting (Fig. 18). To determine binding strengths, his-tagged mutant gp1.2Y28N eluted from the resin at 50-100mM NaCl, gp1.2R37G eluted at 150 mM whereas wild-type gp1.2 required 250mM NaCl for elution (Fig. 13). These data definitively show that the gene *1.2* mutations reduce the affinity of the protein for PifA. The very weak interaction between mutant gp1.2 and PifA is probably the major reason why the mutant gene *1.2* plasmids are compatible with PifA.

d). Mutant PifA K59A interacts with gp1.2, gp10A and FxsA. Analysis of the predicted amino acid sequence of PifA reveals a P-loop, characteristic of an ATP/GTP-binding motif (Saraste *et al*, 1990). A site-specific mutation (K59A) in this P-loop abolishes the exclusion activity of PifA (Wang *et al*., 1999a). To determine whether PifA binds ATP or GTP, a nitrocellulose membrane binding assay was performed by incubating his-PifA with [a-^{32}P]ATP or [a-^{32}P]GTP at 0˚C or at 23˚C in the presence or absence of $MgCl_2$ for 1 hr. No binding was detected. PifA was also mixed with [a-^{32}P]ATP or [a-^{32}P]GTP at 0˚C and cross-linking was attempt using UV irradiation; again no ATP/GTP binding. However, a control assay using T4 DNA ligase revealed ATP binding in both experiments. Either PifA does not bind ATP/GTP or binding is extremely weak and a more sensitive assay is necessary. For example, 5-azido-ATP could be used. Because PifA-K59A remains membrane associated (Fig. 3D) the inactivation of PifA by the K59A

mutation may be that interactions between PifA and one or more of gp1.2, gp10, FxsA, or the unknown cellular target are affected.

To test whether PifA-K59A binds to gp1.2, gp10 or FxsA, immobilized hisPifA-K59A was used as an affinity ligand. Immunoblotting showed that PifA-K59A still binds (Figs. 19 and 20A), showing that the putative ATP/GTP binding site is not essential for the interaction of PifA with gp1.2, gp10A and FxsA. A domain of PifA that includes K59 could be still essential for an activity of PifA required to change or inactivate a cellular function or a conformational change caused by the K59 mutation could prevent PifA from interacting with the cellular target.

e). Gp10 binds to a different region of PifA than gp1.2 and FxsA. In an attempt to localize the regions of PifA that directly interact with gp10A, gp1.2 and FxsA, two his-tagged N-terminal truncation mutants of PifA were created. PifA-del(1-462) contains the C-terminal 255 amino acids and PifA-del(1-180) contains the C-terminal 537 amino acids. Each truncated protein was bound to Ni-NTA agarose and tested for binding gp10A, gp1.2 and FxsA. Gp10A failed to bind PifA-del(1-462) but still interacted with PifA-del(1-180) (Fig. 19). In contrast, both gp1.2 and FxsA bound to PifA-del(1-462) (Fig. 19 and 20A). Thus, gp10A interacts with PifA between residues 181 and 462, and both gp1.2 and FxsA interact with residues between 463 and 717 (Fig 20). [Paragraphs must have more than one sentence] Unfortunately constructs containing C-terminal deletions of

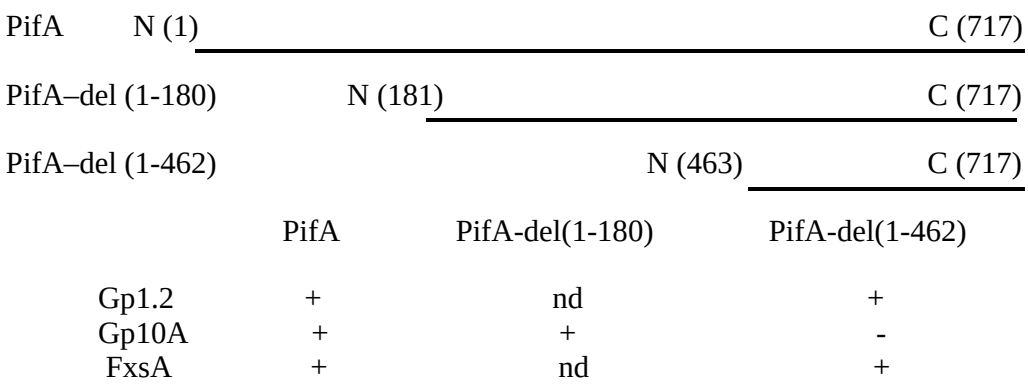
PifA are extremely toxic to *E. coli* and the N-terminal region of Pif could not be tested this way.

The interaction of PifA-del(1-462) with a truncated FxsA containing the N-terminal 111 residues was also examined by co-immunoprecipitation. Truncated FxsA still binds to PifA-del(1-462) (Fig. 20B). This fragment of FxsA contains all transmembrane domains and the small cytoplasmic loop (Fig. 10) and also suppresses F exclusion. Binding of PifA, through its C-terminal 255 residues, to the N-terminal 111 residues of FxsA must play a critical role in suppressing F exclusion. The regions of PifA that have been shown to bind gp1.2, gp10 and FxsA are summarized in Figure 21.

2). Other proteins that interact with T7 gp1.2 and gp10

a). Gp1.2 interacts with *E. coli* PNPase. In the hope that the cellular component targeted by gp1.2 and gp10A during F exclusion could be identified, cell extracts were incubated separately with immobilized his-1.2, GST-1.2 or his-10A. Proteins bound to gp1.2 or gp10A were analyzed by SDS-PAGE. No *E. coli* proteins were found to bind specifically to gp10A. As expected, a 60 kDa protein bound to gp1.2; this protein is likely to be *E. coli* dGTPase. Gp1.2 is known to bind and inhibit *E. coli* dGTPase (Huber *et al*., 1988).

Interestingly ,another *E. coli* protein was found to bind gp1.2 that was identified



	PifA	PifA-del(1-180)	PifA-del(1-462)
Gp1.2	+	nd	+
Gp10A	+	+	-
FxsA	+	nd	+

Figure 21. Summary of the region of PifA interacting with gp1.2, gp10A and FxsA.

+: positive interaction; -: no interaction; nd: not done.

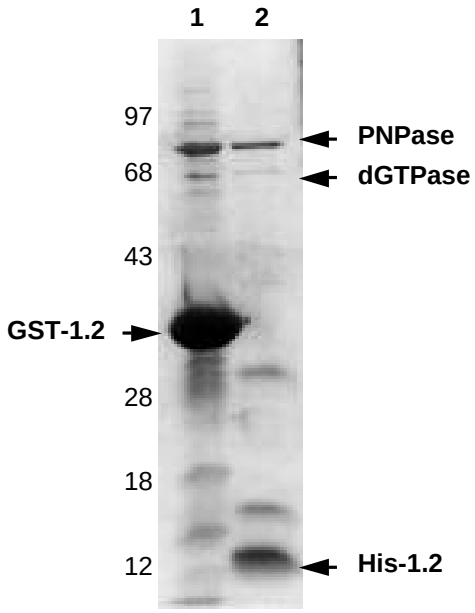


Figure 22. T7 gp1.2 interacts with *E.coli* polynucleotide phosphorylase (PNPase)

Cell extracts were incubated with either GST-1.2 bound to glutathione sepharose or his-1.2 immobilized to NTA-Ni agarose. All proteins that were tightly bound to the resin were eluted with either 10mM glutathione or 250 mM imidazole. Eluted proteins were subjected to SDS-PAGE and then visualized with Coomassie Brilliant blue. Lane 1: GST-1.2. Lane 2: his-1.2.

by N-terminal sequence analysis as polynucleotide phosphorylase (PNPase)(Fig. 22). This result suggests that gp1.2 may affect enzyme activity. Other bands observed are likely to be proteins non-specifically bound to the resin or degradation products of GST-12 in particular. In this binding assay no FxsA was detected, probably because of the low binding affinity of gp1.2 for FxsA and the small quantity of FxsA present in the cell.

To test if gp1.2 affects enzyme activity, gp1.2 and pure PNPase were incubated at both a 1:1 and 5:1 molar ratio at 4˚, 23˚, 30˚ and 37˚C for both 1 and 4 hr, and then assayed for the polymerization and phosphorolysis activities of PNPase using [^{3}H]ADP or ^{3}H-poly A, respectively, under optimal conditions for the enzyme. Surprisingly, gp1.2 neither inhibits nor enhances these activities of PNPase. any significance of the interaction between the two proteins is therefore unclear.

Disruption of *pnp* by Tn*5* increases the plating efficiency of T7 on male cells by at least 6 orders of magnitude at 25˚, 30˚ and 37˚C (Table 11). Generally, the phage only forms pinpoint plaques although at 25˚C and 30˚C plaques are slightly larger. At lower temperatures male cells lacking PNPase appear to be more permissive for T7. Conversely, *pnp* overexpression had no apparent effect. To investigate whether *pnp* null mutations affect PifA synthesis, levels of PifA produced from F and from a multicopy plasmid, in both *pnp*$^+$ and *pnp* null cells,

Table 11. Efficiencies of plating (EOP) of T7 on strains with altered PNPase activity.

	EOP (relative to the isogenic F^- strain IJ511)			
Strains	25 °C	30 °C	37 °C	43 °C
IJ511/F'*lac*	$< 10^{-7}$	$< 10^{-7}$	$< 10^{-7}$	$< 10^{-7}$
IJ511 *pnp::Tn5*	1.09	0.75	0.72	0.66
IJ511 *pnp::Tn5/F'lac*[a]	0.015	0.06	0.014	0.006
IJ511//*F'lac*(pTrcPNP)[b]		$< 10^{-7}$	$< 10^{-7}$	

[a,] Allowed pinpoint plaques only.

[b.] pTrcPNP expressing his-PNPase under *trc* promoter control. Cells were grown in LB to middle log phase and induced with 0.5 mM IPTG for 2 hours before plating.

Table 12. Efficiencies of plating (EOP) of T7 mutants on *pnp* null strains.

	EOP[a]		
Strains	ST16 (Δ*1.2 10*$^{+}$)	MM10 (*1.2*$^{+}$ *10A2D,10A228G*)	MM20 (*1.2 W31C10A2D,10A228G*)
IJ511/F'*lac*	< 10^{-7}	1.8 x 10^{-5}	0.4
IJ511 *pnp::Tn5*	0.9	0.6	1.7
IJ511 *pnp::Tn5/F'lac*[a]	0.02	0.05	0.07

a. Relative to the isogenic F^{-} strain IJ511 at 30 °C.

were examined by immunoblotting. No major change in the level of PifA was detected (data not shown). T7 was also plated on a *pnp* null strain to determine if growth in male cells still required gene *1.2* and gene*10* mutations. No difference was found (Table 12), indicating that the improved plating efficiency in the presence of F is not simply due to a reduced level of PifA, gp1.2 or gp10A. The mechanism of partial suppression of F exclusion by the *pnp* null mutation appears different from that by *strA*, *gyrA43* and *himA* mutations, which simply reduce PifA levels, but how suppression occurs is unclear. PNPase does not appear to be a cellular target for PifA and PNPase may not be directly involved in F exclusion. As T7 RNAs are stable throughout infection, it is possible that one normal function of gp1.2 is to alter the activity of the *E. coli* degradosome (Grunberg-Manago, 1999) by its interaction with PNPase. The absence of PNPase almost certainly changes the physiology of *E. coli* and it may be these changes that account for the increased ability of T7 to grow in the presence of F.

b). Gp1.2 does not interact with gp10A. Although genetic analyses did not suggest a specific interaction between gp1.2 and gp10A, overexpression of nontoxic mutant T7 gene *1.2* permits wild-type T7 to grow in male cells, indicating that the nontoxic gp1.2 not only competes with wild-type gp1.2 for PifA but also prevents gp10 from triggering exclusion (Garcia and Molineux, 1995a). It was thus of interest to ask whether mutant gp1.2 sequesters gp10A from PifA. Gp10A was incubated with both his-tagged wild-type and mutant gp1.2 separately; however,

no binding could be detected. Co-immunoprecipitation also failed to show that gp10A binds to gp1.2 (data not shown). Therefore these two proteins probably do not interact.

3). FxsA interacts with T7 gp10A and gp1.2

PifA has been shown to interact with both gp10A and gp1.2 and their interaction is believed to trigger the abortive infection of T7. Other than through interacting with PifA, FxsA could also mediate protection by binding to gp10A or gp1.2, thereby preventing their interaction with PifA. To test this idea, a cell extract containing FxsA was incubated with resin bound with his-10A or his-1.2 and the eluate was analyzed by immunoblotting. FxsA binds to both gp10A and gp1.2 (Fig. 23). The stability of the interaction between FxsA and gp10A or gp1.2 was measured by dissociating the complex with various concentrations of NaCl. FxsA was eluted from both resins with 100 mM NaCl (data shown as part of Table 10).

Summary of biochemical studies.

PifA interacts with gp1.2, gp10A and FxsA, FxsA with gp1.2 and gp10, but no interaction between gp1.2 and gp10 was detected. Those mutations in gp1.2 and gp10 that allow T7 to escape F exclusion reduce their binding affinity for PifA. These results strongly suggest that the interaction of these proteins triggers F exclusion. The apparent dissociation constant Kd for a gp10A-PifA complex is lower than that of a gp1.2-PifA complex and both Kd values are about the same as

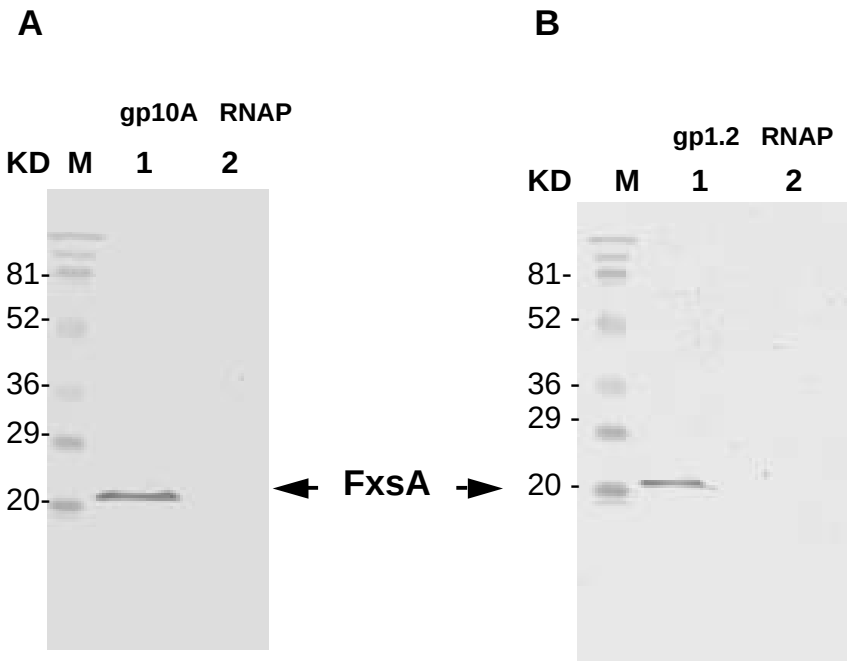


Figure 23. FxsA interacts with gp1.2 and gp10A.

Cell extracts of FxsA were incubated with his-1.2, his-10A or a control his-RNAP resin. Each eluate was subjected to SDS-PAGE and immunoblotted using anti-FxsA serum

the concentrations of these proteins in F^+ cells. Therefore, the Kd values obtained *in vitro* likely reflect physiologically significant interactions *in vivo*. FxsA was also found to bind PifA, gp1.2 and gp10A, supporting the idea that FxsA suppresses F exclusion by interfering with PifA binding its cellular target, gp1.2, or gp10A.

A working model for exclusion of T7 by F.

Based on the results of previous and current studies, a model for exclusion of T7 by F is suggested. PifA exists as an inactive form in the absence of gp1.2 and gp10. After T7 infection, PifA is activated by gp1.2 or gp10 through a direct interaction. The activated PifA then interacts with its cellular target and affects its function. PifA may initially bind to the cellular target prior to phage infection. It is possible that the cell target is a cytoplasmic membrane protein that has the capacity to form an ion channel or that it is a component of binding–protein–dependent transport system; alternatively, it could be the phospholipids that comprise the cell membrane itself. The functional change of this cellular target caused by its interaction with PifA results in an increased membrane permeability due to either an enlargement of ion channels or a structural perturbation of some integral membrane protein or the phospholipid bilayer. Consequently, the membrane potential is lost, the energized membrane collapses, and all cell functions associated with the membrane are therefore affected. FxsA interferes with the interaction between PifA and gp1.2 or gp10, or the interaction between PifA and its cell target by sequestering these parasites' proteins, thereby preventing them

from interfering with the function of the membrane or membrane protein. It is also possible that FxsA helps maintain the integrity of cell membrane. Like the effects of PifA (Wang *et al*., 1999b), that of FxsA is quantitative, its protective function is effective only when sufficient levels of FxsA are present.

Chapter 4. Future Directions

In this study, PifA has been demonstrated to directly interact with both gp10A and gp1.2 *in vitro* with a sufficiently high affinity that suggests comparable interactions also occur *in vivo*. These interactions are likely the initial steps in the process of F exclusion. The membrane localization of PifA and the demonstration that membrane domains of FxsA are essential for suppressing F exclusion provides direct evidence that the cell membrane is the site where gp10A and gp1.2interact with PifA. This evidence further suggests that the cellular target of these proteins is also localized in the membrane.

Because gp1.2 and gp10 interact with FxsA and both are cytoplasmic proteins, the cytoplasmic domain of FxsA is probably the region for this interaction. To determine whether this cytoplasmic loop of FxsA is required in suppressing F exclusion of T7, alanine scanning mutagenesis or selective deletion of a few residues of this domain might prove very informative. Cells synthesizing the mutant FxsA would be tested for their ability to suppress F exclusion. To avoid problems associated with possible changes in membrane topology with mutagenesis, the positively charged residues, arginine 48, lysine 53 and lysine 61, should not be substituted (except for the conservative R->K and K->R changes) or deleted because they are crucial to anchor the second transmembrane segment. A complete replacement of the entire cytoplasmic loop with a different cytoplasmic sequence from a well defined inner membrane protein could also be performed. By

examining the protective function of such FxsA hybrids, it should be easier to judge whether the cytoplasmic sequence is required for suppression of F exclusion. To ensure that any loss of protective function is not due to a change in membrane topology, proteinase sensitivity of FxsA in both spheroplasts and inverted membrane vesicle will be assayed.

Although the protease sensitivity assays performed in this work showed that PifA may have a periplasmic domain, the PifA-PhoA fusion study did not provide any supporting evidence. Whether PifA is an integral or membrane-associated protein remains unclear but it will be necessary to resolve the question in order to understand the mechanistic basis of F exclusion. Knowing whether PifA has a periplasmic domain might also be useful in assessing the normal function of PifA in F biology; it seems unlikely that the autoregulated *pif* operon is maintained by F simply to prevent the spread of T7 and many of its close relatives. Furthermore, several R plasmids are known to interfere with T7 growth (Meynell *et al.*, 1967), perhaps through similar mechanisms to PifA, suggesting that *pif* maybe widespread on conjugative plasmids. It has been shown that the region between residues 181 and 462 contains sequences that are at least partially responsible for the membrane association of PifA. The protease sensitivity assay also suggested that a potential cleavage site may lie between codons 332 and 381 but eight *phoA* fusions to this region did not allow phosphatase activity as judged by blue colonies on XP plates. Although additional fusions could be made it is

hard to justify doing so without additional independent evidence that suggests a transmembrane segment(s) could be present. The K59A mutation of PifA abolishes F exclusion but still allows PifA to bind gp1.2 and gp10, indicating that a domain including lysine 59 may be involved in the interaction with its cellular target or that this residue may be critical for the structural conformation required for the interaction. Regional mutagenesis around lysine59 may help to define this domain or identify other residues that are important for the function of PifA.

Searches of the data bases for proteins with similarity to PifA have not yielded many clues to PifA function. R64, a plasmid that, like F, inhibits T7 growth (Hughes and Meynell, 1973), contains an almost identical gene to F *pifA*. A pathogenicity island in uropathogenic *E. coli* strain 536 codes for a protein closely related to PifA. Predicted proteins of unknown function from various bacterial genera (both Gram negative and Gram positive) are somewhat related to PifA and, interestingly, a predicted protein from a cryptic prophage of *Salmonella typhimurium* also has similarity to PifA. Patches of the PifA amino acid sequence show similarity to a number of ABC transporters and other ATP-binding proteins, similarities are centered around the predicted P-loop of PifA but neither ATP nor GTP binding could be demonstrated. Perhaps, as suggested earlier, use of a photo-activated cross-linking ATP derivative would reveal an interaction.

By using affinity chromatography no *E. coli* protein was found to interact with PifA and gp10. This indicates that any interaction between a host protein and

those of the parasites is weak. A more sensitive assay might reveal an interaction. Chemical cross-linkers, e.g., dithiobis-(succinimidyl propionate) is a commonly used thiol-cleavable reagent (Gunzburg *et al*., 1989) that could be employed. Crosslinks could be made *in vivo* by incubating the reagent with cells overproducing his-tagged PifA, gp1.2 or gp10 or *in vitro* by incubating the cross-linker with cell extracts prior to affinity chromatography. A yeast two-hybrid screen with PifA, gp1.2 or gp10A as bait to screen an *E. coli* genomic library is another approach to find interacting proteins. However, because PifA is a membrane associated protein, it likely binds to its cellular target before infection by T7. Thus it would be initially more useful to search for a PifA-interacting component.

It has been suggested that the cellular component targeted by PifA and gp1.2 or gp10 is likely localized in the membrane and may be crucial for viability. However, there is no *a priori* reason that the component must be protein and it could be the membrane itself. Phospholipids could be the target. If this idea is correct it might explain why mutations affecting the target of PifA and gp1.2 or gp10 were not isolated in several mutant searches.

The *E. coli* membrane contains 70-80% phosphatidylethanolamine(PE), 15-20% phosphatidylglycerol (PG) and 2-5% cardiolipin (CL). Strains lacking PE or CL are viable but the former need 50 mM $MgCl_2$ in order to grow. Strains lacking PG have also been isolated (Kikuchi *et al.,* 2000) and a strain was made in which

the levels of PE and CL can be controlled by IPTG (Heacock and Dowhan, 1989). *E. coli* mutants containing an altered phospholipid composition could be tested for their ability to support T7 growth and, if so, whether F exclusion still functions. In principle F exclusion should be affected if the strain lacks the type of phospholipids that is targeted by exclusion system.

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Vita

Xiaogang Cheng, male, was born in Lanzhou, Ganshu province, China on Dec. 11, 1962. He entered the School of Veterinary Medicine, Ningxia Agricultural University in Nixia, China in 1980. He received D.V.M. in July 1984. After two years as a technician in the Center for Control of Disease in Yinchuan, he began his graduate study in the National Institute for Control of Veterinary Bioproducts & Pharmaceuticals, Beijing, China in 1986. He graduated with a Master of Science degree in 1989 and then worked as a research assistant in the department of virology of the same institute for five years. In the fall of 1994 he came to the United States of America to start his study in the Graduate School of University of Texas at Austin.

Permanent address: 1426 Carriage Bridge Trail,
Ballwin, MO 63021

This dissertation was typed by the author.